Universidade de Aveiro Departamento de Biologia
Ano 2016

Ricardo Jorge da Costa

Avaliação de fatores que determinam as respostas genotóxicas a pesticidas - o lagostim *Procambarus clarkii* como espécie modelo

Unravelling the factors determining the genotoxic responses to pesticides - the crayfish *Procambarus clarkii* as a model species

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Ecologia Aplicada, realizada sob a orientação científica da Doutora Sofia Isabel Antunes Gomes Guilherme, Investigadora de Pós-doutoramento do Departamento de Biologia da Universidade de Aveiro, e do Doutor Mário Guilherme Garcês Pacheco, Professor Auxiliar c/ Agregação do Departamento de Biologia da Universidade de Aveiro.

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palavras-chave

Herbicidas, Viper®, dano genético, fatores determinantes de dano, *Procambarus clarkii*

resumo

Os mecanismos de resposta e adaptação dos organismos à presença de contaminantes, assim como o subsequente reflexo destes no sucesso da população estão ainda pouco estudados no contexto da toxicologia ambiental. Tendo em conta que a integridade do material genético (ADN) é vital para os seres vivos, torna-se importante analisar as eventuais estratégias de ajuste genético desenvolvidas por espécies bem-sucedidas, permitindo-lhes sobreviver num ambiente contaminado, nomeadamente por pesticidas.

Deste modo, os principais objetivos do presente estudo baseiam-se na compreensão da influência de fatores como "População" e "Género" nas respostas genotóxicas do lagostim *Procambarus clarkii*, quando exposto a um herbicida de uso comum (Viper®) e também a um modelo genotóxico (etil metanosulfanato - EMS), em duas populações distintas (uma recolhida num local considerado como referência – P1, e a outra proveniente de um local com historial de contaminação por pesticidas – P2). Tentou-se ainda identificar os mecanismos de dano envolvidos nos eventuais ajustes e/ou vulnerabilidades demonstradas por *P. clarkii.* Adicionalmente, pretendeu-se contribuir para a avaliação do risco ambiental relacionado com a utilização de pesticidas, contribuindo assim para a identificação das estratégias desenvolvidas pelo lagostim *P. clarkii* no sentido de melhor lidar com o dano genotóxico.

Assim, espécimes de lagostim de cada população (P1 e P2) foram divididos em 4 grupos, cada um com 12 indivíduos (6 machos e 6 fêmeas; n=6), correspondendo a um controlo negativo, a um genotóxico modelo (EMS 5 mg L^{-1}) e a duas concentrações ambientalmente relevantes do herbicida Viper®, 20 μgL^{-1} (V1) e 40 μgL^{-1} (V2).

A avaliação do dano genético foi feita recorrendo a uma versão melhorada do ensaio cometa, permitindo ainda, desta forma, a deteção de dano do tipo oxidativo. Os resultados mostraram a genotoxicidade do herbicida Viper® para a espécie não-alvo *Procambarus clarkii*. A utilização de um modelo genotóxico (EMS) permitiu observar uma aquisição de uma proteção/vulnerabilidade não específica em relação a danos genéticos. Os organismos da população previamente exposta revelaram uma susceptibilidade maior à pressão genotóxica não específica posta por Viper®, enquanto que em relação à oxidação do ADN a mesma população mostrou uma maior capacidade para lidar com este tipo de dano. Estes efeitos referentes ao historial de exposição só foram evidentes em machos e em relação ao Viper®.

Globalmente, e considerando a avaliação genotóxica como um todo, foi demonstrada a influência de fatores como "População" e "Género", destacando a importância de se considerar as diferenças nos antecedentes fisiológicos dos organismos para a avaliação ecogenotoxicológica, permitindo a elaboração de abordagens de monitorização ambiental mais plausíveis e holísticas.

keywords Herbicides, Viper®, genetic damage, factors determining damage, *Procambarus clarkii*

abstract

The way organisms may respond and adapt to the presence of contaminants and the subsequent repercussion on the success of the population are still poorly explored issues in the context of environmental toxicology. Knowing that the integrity of the genetic material (DNA) is vital for living beings, it is important to shed a light on eventual genetic adjustment strategies developed by well-succeeded species, in order to cope with, namely, pesticide environmental contamination.

Thus, the major goals of the present study were to understand the influence of factors as "Population" and "Gender" in the genotoxic responses of the crayfish *Procambarus clarkii*, when exposed to a widely used herbicide (Viper®) and also to a model genotoxicant (ethyl methanesulfonate - EMS) in two distinct populations (one collected from a reference site – P1, and the other from an historically impacted site – P2). It was also sought to identify the damage mechanisms involved in eventual positive adjusts and/or vulnerabilities demonstrated by *P. clarkii.* Moreover, this study contribute to the evaluation of the environmental risk related to the utilization of pesticides and to contribute to the identification of strategies displayed by the crayfish *P. clarkii*, in particular, to cope with genotoxic damage.

Therefore, crayfish specimens from each population (P1 and P2) were then divided into 4 groups, each with 12 individuals (6 males and 6 females; n=6), corresponding to a negative control, a model genotoxicant (5 mg L^{-1} EMS) and two environmentally relevant concentrations of the herbicide Viper®, 20 μgL^{-1} (V1) and 40 μgL^{-1} (V2).

The improved version of the comet assay was used to assess the genetic damage, allowing, this way, the detection of oxidative lesions. The results proved the genotoxicity of the herbicide Viper® to the non-target species *Procambarus clarkii.* The use of a model genotoxicant (EMS) allowed us to observe an acquisition of a non-specific protection/vulnerability in relation to genetic damage. Organisms from the previously exposed population revealed a higher susceptibly towards the non-specific genotoxic pressure posed by Viper®, while in relation to DNA oxidation the same population showed an increased ability to deal with this type of damage. Furthermore, these effects of the exposure history were only evident in males and in relation to the agent Viper®.

Overall, and considering the genotoxic evaluation as a whole, the influence of factors as "Population" and "Gender" was demonstrated, highlighting the importance of consider differences on the organisms' physiological background for ecogenotoxicological-based environmental health assessment, permitting the elaboration of more plausible and holistic approaches.

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1. Introduction

Several species are able to survive in aquatic environments containing high levels of multiple anthropogenic pollutants or/and natural toxins. Various estuarine and freshwater ecosystems around the globe are contaminated with aromatic hydrocarbons (AH), including polychlorinated biphenyls (PCBs), polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), polycyclic aromatic hydrocarbons (PAHs), metals, pesticides and their respective metabolites, as well as other compounds (Wirgin and Waldman, 2004).

The occurrence of these pollutants in the environment offers the opportunity of studying the action of natural selection and selective agents on biota. In addition, the knowledge concerning the presence and effects caused by pollutants may help to illustrate the processes involved in early stages of speciation (Williams and Oleksiak, 2008). But considerations of a less theoretical nature also highlight the importance of investigating the evolution of safeguard mechanisms. This shift of emphasis has great bearing on the type of research that must be performed to detect, for example, genotoxicity in natural populations. This kind of approach also highlights the importance of studying populations instead of singular individuals (Depledge, 1994), revealing the selection mechanisms acquired (Williams and Oleksiak, 2008), for instance, concerning the natural sensitivity due to a contamination history (Sevatdal and Horsberg, 2003).

One of the most common pollutants found in aquatic habitats are pesticides (Aktar et al., 2009). These compounds can be classified based on: their chemical structure (for example, carbamates, organophosphates, organochlorines, and pyrethroids), their target (for example, insecticides, herbicides, fungicides, rodenticides, molluscicides, nematicides and acaricides) and their mode of action (for example, acetylcholinesterase inhibitors, and calcium channels inhibitors). Boxall et al. (2002) and Dabrowski et al. (2005) demonstrated that herbicides affect the ecosystem at several levels, since the parental compound or the breakdown products (or metabolites) may be accumulated in flora, fauna, sediment and water, occurring mainly due to spray drift or by run-off and consequent soil leaching (Belenguer et al., 2014, Kopciuch et al., 2004, Masia et al., 2013). In particular, when herbicides are released into aquatic habitats, several

(direct) toxic effects on aquatic biota may occur, such as: cancerous tumours and lesions, reproductive inhibition, disruption of endocrine system, cellular and DNA damage, transgenerational effects, and, ultimately, death (Helfrich et al., 1996). These effects may vary with the intensity and duration of the exposure, typically reducing organism's abundance (by increased mortality or reduced fecundity). Due to their impact, these effects are frequently studied, in part, because of the predictive criteria used to estimate risk and establish permissible levels of contamination (Fleeger et al., 2003).

Biota from a given habitat often exhibit a wide range of adjustment strategies to specific pesticides (e.g. insecticides and herbicides target specific organisms in an interacting community) which are often related with their habitat, contamination history and life stages. These influencing factors are not commonly studied, despite being signalized as highly important when performing a population study. Nonetheless, direct sublethal effects, such as behavioural impairment or physiological stress, are also considered as important issues that must be included in this kind of studies. Thus, these effects may affect sensitive species, altering their competitive interactions, which may lead to their disappearance in some environments. Considering this, toxicants may directly influence 'keystone' or 'foundation' species (Bruno and Bertness, 2001). Similarly, disturbance rates or resource availability may be influenced by contaminants, which may in turn modify important ecosystem functions (e.g. decomposition rates, oxygen dynamics and nutrient cycling) (Fleeger et al., 2003).

Experiments on the toxic potential of pesticides have been performed considering an amply variety of organisms, from microorganisms to mammals (Jha, 2008). The usage of crustaceans as model organisms (e.g. *Daphnia magna*, *Astacus leptodactylus, Procambarus clarkii*) in ecotoxicology (Benli et al., 2007; Jha, 2008; Suárez-Serrano et al., 2010a) has become very common since these organisms can provide information on stress responses induced by short-term changes in the surrounding environment, whilst having several other important characteristics. Among aquatic organisms, crustaceans play a key-role in the environment for their intermediate position in the food web and also for their wide distribution and high abundance. Also, crustaceans support the usage of a variety

of biomarkers (e.g. cytological, genetic, biochemical, or molecular) (Monserrat et al., 2007) designed to assess the environmental quality. These biomarkers act as early warning signals towards the presence of potential toxic compounds and/or substances, and are useful tools to assess both the occurrence of exposure and/or their effects, consequently, providing information about toxicant bioavailability (Picado et al., 2007).

Alterations caused by pollutants can modify the genetic information in a variety of organisms in terms of integrity and stability, which are essential for maintaining cell homeostasis. If these genetic modifications are not repaired, they may lead to mutations and possibly disease (Helleday et al., 2007). Genetic information is coordinated by the main molecule in living organisms, the DNA. This molecule contains the instructions used in the growth, development, function and reproduction of all known living organisms and many viruses. In fact, it has been estimated that an individual cell can suffer up to one million DNA changes per day (Lodish et al., 2004) and the very process of DNA replication during cell division is prone to error (Helleday et al., 2007).

1.1 Genotoxic potential - direct or indirect effects

Genotoxic potential of pesticides is of huge concern, mainly due to the fact that the key role of DNA in the organism implies that genotoxic damage may escalate into severe problems at an intracellular level, resulting in highly negative consequences (Bolognesi et al., 2003; Nagarathna et al., 2013; Rahman et al., 2002; Robertson, 2001; Straalen, 2003).

Genotoxicants can exert direct or indirect effects on DNA. Some examples of indirect effects are the mistimed events activation, inducing mutations, among others. Permanent and heritable changes can affect either somatic or germ cells, being the latter able to transmit these alterations to future generations (Kolle, 2012). On the other hand, toxicity in somatic cells may cause a variety of toxic effects on the organism (such as cancer and/or death). Alterations in germ cells may have repercussions at the population scale, since they may compromise the species genetic viability. However, some acquired hereditable adjustments may

lead to a population with increased resilience to adverse conditions, such as environmental toxicity (Klerks and Moreau, 2001). Moreover, indirect effects may also result into interactions with non-DNA targets, essentially through lipid peroxidation and protein adducts formation.

As mentioned above, mechanisms of genotoxicity may be direct, reflecting thus straight interactions with DNA (Kirsch-Volders et al., 2003), with non-desirable consequences as well (Figure 1).

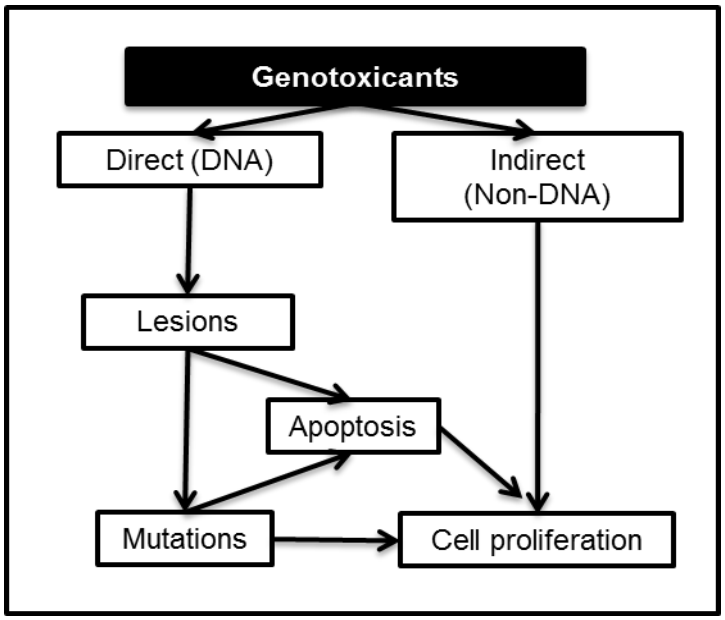

Figure 1. Overview of mechanisms by which direct and indirect acting genotoxicants affect the cell (Adapted from Kirsch-Volders et al., 2000).

1.1.1 DNA damaging events

Genotoxicants can cause a variety of damaging events in DNA. This DNA damage can result into single and double-strand breaks, loss of excision repair, cross-linking, alkali-labile sites, as well as structural and numerical chromosomal aberrations (Sancar et al., 2004).

DNA single-strand breaks (SSBs) are among the most frequent DNA lesions, arising directly from damage to the deoxyribose moieties or indirectly as intermediates of DNA base excision repair (BER). Left unrepaired, SSBs are a

major threat to genetic stability and cell survival, accelerating mutation rates and increasing levels of chromosomal aberrations (Helleday et al., 2007). Additionally, double strand breaks (DSB) can lead to mutations or to larger-scale genomic instability through the generation of dicentric or acentric chromosomal fragments. Such genome changes may have tumorigenic potential and, in other instances, DSBs can be sufficient to induce apoptosis. Because of the threats posed by DSBs, eukaryotic cells have evolved complex and highly conserved systems to rapidly and efficiently detect these lesions, signalizing their presence and bring about their repair (Jackson, 2002). Fortunately, there are systems to repair DSBs. These lesions are mainly repaired by either homologous recombination repair (HRR) which comprises a series of interconnected pathways that function in the repair of DSBs and interstrand crosslinks (ICLs) (Van Gent et al., 2001).

If the DNA suffers an attack during replication, alkali-labile sites and point mutations can occur; consequently, chromosomal aberrations (clastogenic) lead to sections of the chromosome being deleted, added, or rearranged numerical aberrations (aneuploidy) can be either loss or gain of chromosomes per cell and can be lethal or cause a genetic disease (Nagarathna et al., 2013; Rastogi et al., 2010).

Chromosome aberrations can either be structural, leading to the loss of acentric chromosomal fragments (clastogenic), or numerical, generating loss of chromosomes (aneugenic). These aberrations can be caused by a clastogenic chemical, which is a mutagenic agent that can cause disruption or breakages of chromosomes, leading to the deletion, addition of rearrangement of certain sections of the chromosome. The clastogenic or aneugenic effects from the genotoxic damage will cause an increase in frequency of structural or numerical aberrations of the genetic material. It is a fact that unrepaired or misrepaired DSBs can lead to mutations, chromosome rearrangements, cell death and cancer (Dronkert et al., 2000; Dudaš and Chovanec, 2003).

1.1.2 Mechanisms of DNA repair

DNA repair mechanisms occur in order to protect the integrity of the molecule and cell viability. Depending on the specific class of DNA lesions, one or more DNA repair pathways may become active (Zheng et al., 2005).

In case of DNA damage, the most common mechanism is called direct repair which acts by removing or reversing the DNA lesions by a single enzyme reaction in a basically error-free manner and with high substrate specificity (Mourgues et al., 2007; Sedgwick et al., 2007).

When the damage is detected during the cell cycle, the prevalent repair mechanism is the base excision repair (BER), a mechanism that protects the cell from deleterious effects of endogenous DNA damage induced by hydrolysis, reactive oxygen species (ROS) and other intracellular metabolites. Moreover, it also may remove lesions caused by ionizing radiation and alkylating agents (Rastogi et al., 2010). The main enzymes in BER are DNA glycosylases and AP endonucleases (apurinic/apyrimidinic endonuclease), which act in the following order: the DNA glycosylases remove the damaged base, where after the remaining a-basic site is further processed by AP endonucleases (Essers et al., 2000; Van Steeg, 2009). If flawed insertions and or bases are detected, mismatch repair (MMR) enters in action (Natarajan, 1993). These errors occur during DNA replication and MMR is a strand-specific repair. During DNA synthesis, the daughter-strand may include incorrect bases, such as G/T or A/C. To repair these mismatches in the correct manner, it is very important to discriminate between the newly synthesized (mismatched) strand and the parental strand. The first step in MMR is the detection of the deformity caused by the mismatch. Thereafter, the template and the non-template strand are determined and the incorrectly incorporated base is removed and replaced by the correct nucleotide. During this process, the mismatched nucleotide and some of the newly synthesized DNA strand can be removed and replaced (Natarajan, 1993).

In the event of lesions being originated from exogenous sources, like UV light or genotoxic chemicals producing bulky adducts and DNA cross-links, the cell triggers the nucleotide excision repair (NER), a pathway that is involved in the removal of several kinds of DNA lesions (Hanawalt et al., 2003). NER consists of

two different sub-pathways: global genome repair (GGR) and transcription-coupled repair (TCR). These two sub-pathways only have a difference in the first step of DNA damage recognition (Hoeijmakers, 2001).

1.1.3 Methodologies for the evaluation of genetic damage

The use of genotoxic endpoints allows the evaluation of DNA damage, therefore establishing a relationship between the exposure to genotoxicants and presumable effects in organisms. A variety of techniques have been developed to assess the chemicals' potential to cause DNA damage. Moreover, the analysis of DNA alterations is considered a highly suitable approach for the evaluation of exposure to low concentrations of genotoxicants (Scalon et al., 2010), namely certain herbicides (Frenzilli et al., 2009). During the last decades, there has been a continuous development in terms of responsive genotoxic biomarkers, especially in aquatic organisms (Hayashi et al., 1998).

One commonly used technique is the erythrocytic nuclear abnormalities (ENA) assay, that consists in the detection of micronuclei (originated from chromosome fragment or a whole chromosome which during the cell division lags behind) (Fenech, 2000) and other nuclear abnormalities in nucleated mature erythrocytes (Pacheco and Santos 1997). These abnormalities, firstly identified by Carrasco et al. (1990), are normally categorizes as: kidney shaped nuclei (K), lobed nuclei (L), binucleate or segmented nuclei (S) and micronuclei (MN). These nuclear deformations are signals of chromosome breakage (clastogenicity) or loss and mitotic spindle apparatus dysfunction (aneugenicity) (Fenech, 2000; Stoiber et al., 2004).

Another way to evaluate genotoxic damage is analyzing the occurrence of sister chromatid exchanges (SCE). They appear during cell replication, when a chromosome duplicates its genetic material, forming a pair of chromatids attached at the centromere. Chromatids can even exchange seemingly identical segments of DNA without known alterations of cell viability or even its functions (Wilcosky and Raynard, 1990). There are two models concerning the formation of SCE: the recombination model and the replication model, consisting in chromatid exchanges

as part of a post-replication repair process, and recombination during the DNA replication (Wilcosky and Raynard, 1990), respectively.

By last but not the least, the comet assay is a simple, versatile and sensitive assay. This technique primarily measures DNA strand breakages in single cells. It was firstly developed by Östling and Johansson in 1984 and modified by Singh *et al,* in 1988. Its use has increased in different contexts as clinical applications, human monitoring, radiation biology and genetic toxicology (Cotelle and Ferard, 1999). The application of the comet assay has also given important contributions to the ecotoxicology field. Besides the presented advantages, this method provides the opportunity to study DNA damage (including oxidative damage) and repair in different cell types without prior knowledge of karyotype and cell turnover rate (Jha, 2008). The comet assay has been applied to several species, already used in biomonitoring and ecogenotoxicological studies, and has proven to be a sensitive system for screening the genotoxicity of chemicals and complex mixtures (Cotelle and Ferard, 1999).

1.2 Factors that may influence the occurrence of genotoxicity

Factors that influence the expression of genotoxicity in organisms are relatively common (Bleich, 2006). These are therefore denominated as genotoxic factors. Three factors which are present along this study are: population (relatively to organisms' life history), gender and treatment (associated to the different concentrations of compounds that the organisms were exposed).

The factor population is interlinked with the historical exposure of organisms to specific compounds that may result in a variety of responses against the same or even other pollutants (Brausch and Smith, 2009; Weston et al., 2013)

Gender factor is directly related with differential responses obtained from males and females, when subjected to the same conditions.

Last but not least is the treatment factor, here defined as the effect that different concentrations of specific compounds may influence different responses in tested organisms.

1.2.1 The influence of gender on genotoxic responses

Gender differences can have a major effect in genotoxicity, because males and females present several differences in terms of behavior, physiology, metabolism, lifestyle and life expectancy.

There is some tendency in the literature towards showing females as more resistant to genetic damage and with longer life spans. This difference in resistance may cause an earlier death of the males, which holds for most cases, but can also be the result of sex reversal in males as shown for *Crangon franciscorum* (Gavio et al., 2006) or even the sex ratio being more favorable to females.

Genotoxic gender-based responses have already been explained by differences in regulated hormones in zebrafish (Shao et al., 2012) where an alteration in their concentrations can cause an increase concentration of ROS (Halliwell and Gutteridge, 1999). However, there is a lack of studies providing verifiable evidence related to gender DNA-related responses, as stated by Weber et al. (2013).

1.2.2 How the environmental contamination historical may influence organism's genotoxic responses?

Organisms with a different background concerning environmental exposure tend to present a variety of responses, caused by different contaminants (Fleeger et al., 2003). These responses can be direct, affecting the organism abundance, or indirect, leading to changes in behaviour, competitive interactions and alterations, for instance, in predator-prey relations (Fleeger et al., 2003). As a result of the chemical pollution and habitat destruction, both extinction and the evolution of new species, which have been always a normal part of life, are now increasing (Bickham, 2000).

Environmental contamination can provoke the decrease of the number of individuals or direct the development of adaptions (Diekmann et al., 2004) in populations inhabiting contaminated sites (Brausch and Smith, 2009). Populations

in contaminated sites can either adapt or acclimate to pollutants (Ownby et al., 2002).

Populations previously exposed to a specific contaminant and with high genetic variability can develop more protections against environmental contamination than a population with low genetic variability and with zero history of contamination (Klerks, 1999).

1.2.3 Tolerance and resistance

Populations with different origins can have different responses when in contact with genotoxicant agents (De la Sienra et al., 2003; Klobučar et al., 2012). They can be more susceptible to damage if from a contaminated site (Biggs et al., 2007), and more resilient to this damage if they come from a non-contaminated site (Regoli, 2000). However, in other cases, organisms previously exposed to contamination may develop an adjustment, due to the occurrence of previous expositions to, for example, pesticides (Brausch and Smith, 2009; Weston et al., 2013).

Tolerance and resistance are two different concepts. Whilst resistance is related to heritable features or the innate ability to resist the adverse effects of some agents, tolerance is the ability to limit the damage caused by external agents such as physical and chemical factors (pollutants). These two concepts can illustrate the processes involved in early stages of speciation (Williams and Oleksiak, 2008). Frequently, only the end-product of speciation is observable, but, due to anthropogenic effects, an increase of pollution has occurred allowing us to observe populations with a higher tolerance to pollution (Klobučar et al., 2012).

Population studies which demonstrated tolerance to various contaminants also revealed that, in some cases, such phenomenon can be restricted to certain life history stages (Taylor and Weiss, 2010). Examples of tolerance development include increased metallothionein levels in the presence of certain metals (Sanders et al., 1996), and increased activity of metabolizing enzymes in response to chemical contaminants (Gonzalez and Nebert, 1996).

On the other hand, resistance allows populations to inhabit and survive environments that become contaminated, despite the occurrence of substantive toxic effects, as a consequence of natural selection, resulting in genetic adaptation to the contaminant effects. This can lead to hormesis - the stimulation of the organism performance occurring when exposed to toxic or harmful agents (Forbes, 2000). It remains unclear how commonly such adaptations occur (Klerks and Moreau, 2001). These authors also found a negative relationship between the heritability of contaminant resistance and the number of contaminants to which the organisms were simultaneously exposed. However, considerations other than the presence of additive genetic variance point toward selection for resistance to contaminants becoming less effective when more contaminants are involved. This indicates that natural selection will be unlikely to save populations that are impacted by a large number of different contaminants. Resistance mechanisms like metabolic detoxification, reduced neural sensitivity, and reduced penetration of contaminants are important defense mechanisms, particularly against pesticides (Brausch and Smith, 2009).

1.2.4 Impact of the life stage in ecotoxicological studies

The default choice in ecotoxicological studies is starting to be the most sensitive life stages of the target species, since they can provide an easy, fast and precise toxicity test, with the added advantage of being cheaper in terms of maintenance (Mohammed, 2013).

The results obtained from these early stages are ecologically relevant and used in predicting effects of pollutants in the field (Hardersen and Wratten, 2000; Hoang and Klaine, 2007; Kennedy et al., 2006). In this context, toxicity tests with embryos, larvae and juveniles are deemed as being particularly valuable (Gopalakrishnan, 2008; Schmieder et al., 2000; Zhang et al., 2012). Evidence from pesticide-resistance studies in insects (where the larval and adult stages are very different physiologically) indicates that resistance in different life stages is often highly dependent of the stage (Klerks and Moreau, 2001). On the other hand,

cases like *Asellus militaris* (a crustacean) present adults as more sensitive than juveniles.

To counter point the present tendency, adult organisms are used in this study. They were chosen due to the possibility of clarify gender-specific responses to a given compound, which would not be possible by using juveniles. Differences that are normally associated when comparing adults and juveniles are: surface area/volume ratio; developed homeostatic mechanisms, and the development or organ systems, among others (Mohammed, 2013).

1.2.5 Age related responses to genetic damage

Despite the importance of the life stage, age was also demonstrated to have a major role concerning the occurrence of damage, particularly of the oxidative type, since it is related to the loss of physiological functions (Hamilton et al., 2001).

This tenet is reinforced by Akcha et al. (2004) and Hamilton et al. (2001) studies, which results state that the increase of DNA damage is related to a decline in the ability of cells to repair. It was also stated by Hamilton et al. (2001) that older individuals may accumulate in their organs different substances that increase the potential of some compounds, enhancing in a way that predisposes genetic damage.

In some cases, the opposite also occurs and young individuals can display higher genetic damage than adults. This was observed in a study performed by Pellegri et al. (2014), where *Daphnia Magna* (crustacean) with 24 hours of life displayed higher damage than individuals with 48 hours. A possible explanation to this occurrence is the fragility of the carapace of younger individuals, resulting in higher mechanic stress, thus leading to an increase in damage.

1.3 *Procambarus clarkii* as a model organism to test factors that might influence pesticide genotoxicity

Freshwater crayfish are solitary and sedentary bottom dwellers known to accumulate pollutants in their tissues. They are widely distributed and relatively easy to collect (Anderson et al., 1997; Khan et al., 1995; Schilderman et al., 1999).

Procambarus clarkii has a major importance in almost all of the ecosystems in which it is present, mainly due to its resistance to pollutants and the capacity to accumulate them in their tissues. Moreover, its position in the middle of the trophic chain turns this species into a vector of contamination to other trophic levels (Gherardi and Lazara, 2006). All these factors contribute to elect *P. clarkii* as a good bioindicator of metals, organic contaminants and pesticides (Desouky et al., 2013; Kouba et al., 2010; Vioque-Fernández et al., 2007a, 2007b, 2009a and 2009b).

This organism possesses an external morphology similar to other crayfish specimens (Figure 2).

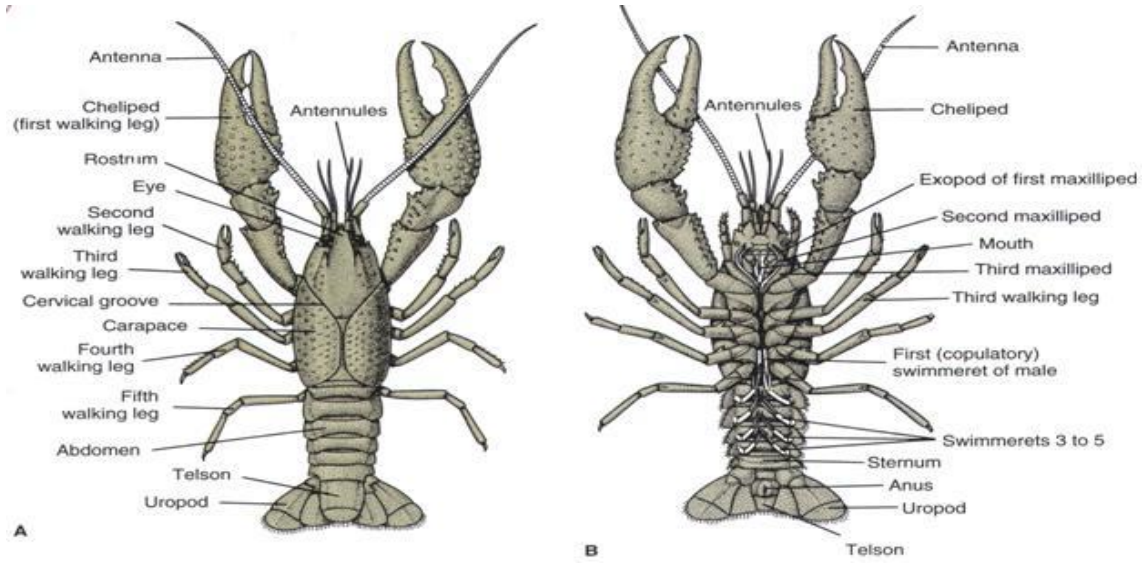

Figure 2. External morphology of *Procambarus clarkii* (adapted from Hobbs and Jass, 1989). A- dorsal view; B- ventral view.

In what concerns its suitability to be used in genotoxicity studies, it was demonstrated that this species can provide important information on stress responses induced by short-term changes in the surrounding environment (Suárez-Serrano et al., 2010a). However, there is a lack of information concerning the use of freshwater crayfish in environmental pollution monitoring.

1.3.1 *Procambarus clarkii* range of distribution

This species is native to southern USA and northern Mexico (Hobbs, 1984; Hobbs et al., 1989). The invasive success of *P. clarkii* has been mainly attributed to its high environmental tolerance and fecundity (Gherardi et al., 2000; 2002; Holdich et al., 2009; Suárez-Serrano et al., 2010a).

Currently, due to anthropogenic actions, it can be found in the following geographical areas: Africa, South and Central America, Asia Continental, Pacific, Caribbean and Europe (Hobbs et al., 1989; Holdich, 1987; Huner and Avault, 1979).

In Europe, this species occurs in Portugal, Spain and France. In the case of Spain, the first introduction took place in 1973 in Badajoz area (Habsburg-Lorane, 1979), and in 1974 it has been introduced in the province of Seville (Gaudé, 1984).

1.3.2 The advantages of sexual dimorphism in an assessment of genotoxicity

This species presents sexual dimorphism, partially based on the disproportion of the pincers (which are bigger in males) and also a number of small morphological adaptations in males that facilitate mating (Dawes, 1981; Holdich and Lowery, 1988; Marshall and Williams, 1972; Taketomi et al., 1990).

The correlation between sexual dimorphism and genotoxicity may be difficult, since a wide range of variables must be taken into account (Fortoul et al., 2004; Iarmarcovai et al., 2007). Since *P. clarkii*'s sexual dimorphism and genotoxicity is not a well-discussed theme, and even when both genders are present in certain studies it is only for the purpose of assessing differences in parameters like LC_{50} (Abdel-kader, 2016), there is a need to improve the knowledge concerning this.

1.4 Genotoxic risk of Viper® - an unexplored issue

Since several herbicides showed to be genotoxic to aquatic organisms (Guilherme et al., 2012: Guilherme et al., 2014a; Yüzbaşioğlu et al., 2009), and the genotoxicity of Viper® remains largely unknown, the present work intends to shed a light concerning this issue.

The commercial formulation Viper®, containing penoxsulam 2-(2,2-Difluoroethoxy)-N-(5,8-dimethoxy[1,2,4]triazolo[1,5-c]pyrimidin-2-yl)-6-(trifluoromethyl) benzenesulfonamide as the active ingredient, is a post-emergence herbicide commonly applied in paddy fields, by terrestrial or aerial drift, for the selective control of weeds (Roberts et al., 2003). This herbicide appears as an oil dispersion, containing 97.81 % of other ingredients (not specified), including also an adjuvant and methanol in its constitution (Dow AgroSciences, 2002).

This herbicide acts through the inhibition of the acetolactase synthase (ALS), an enzyme which catalyses the first step in the biosynthesis of branch amino-acids (i.e. valine, leucine and isoleucine), typical of microorganisms, fungi and plants, that is absent in animals. The active ingredient, penoxsulam, has been found to dissipate rapidly in paddy waters, being the most important process the photo-degradation (Jabusch and Tjeederma, 2006a), and relatively rapidly in paddy soil where microbial degradation is the prominent process (Jabusch and Tjeederma, 2006b). It has eleven major breakdown products, six of them considered as products of toxicological concern. However, none of the eleven have been identified as having a higher toxicity potential than the parent compound (penoxsulam) (Washington State Department of Ecology, 2012).

Penoxsulam became one of the most widely used herbicides in rice crops in the world (Jabusch and Tjeerdema, 2005), increasing its potential interest to be studied. Despite this, there is a lack of information concerning its oxidative potential and genotoxic differences. Considering populations, only a few studies have been performed, as the example of Murussi et al. (2014).

1.5 Study framework and dissertation goals

Although populations of the same species appear as morphologically equal, their different backgrounds or origins may cause dissimilar responses to environmental stressors. This assumption served as a basis for the present study, where two populations of the crayfish *Procambarus clarkii*, with different origins and subsequent dissimilar contamination history, were subjected to a laboratory exposure to the herbicide Viper®, as well as to a model genotoxicant (EMS).

The region of Vouga Lagoon System (Portugal), more precisely in Salreu, has a history of rice culture, where Viper® is frequently used. The population of *P. clarkii* captured in this site, defined as historically impacted due to recurrent presence of this herbicide, was compared to one collected from the Minho river (without pesticide application history), in order to identify the possible differences between them.

Thus, the main goals of the present study were: (1) to understand the influence of factors as “Population” and “Gender” in the genotoxic responses of the crayfish *P. clarkii*, when exposed to a widely used herbicide (Viper®) and also to a model genotoxicant (EMS); (2) to identify the damage mechanisms involved in eventual positive adjusts or vulnerabilities demonstrated by these organisms; (3) to contribute to the understanding of the environmental risk related to the utilization of pesticides, contributing to the identification of strategies displayed by the crayfish *P. clarkii* to cope with genotoxic damage and, finally, (4) to provide scientific data able to improve agro-industry managing practices, in order to mitigate the agrochemical effects in aquatic environment.

This study was carried out through the implementation of a short-term (7 days) laboratory experiment.

2. Material and Methods

2.1 Chemicals

The experiment was conducted using the commercial formulation Viper®, distributed by Dow AgroSciences LLC (Portugal). It contains 2.4 g L^{-1} of penoxsulam, Its formulation is oil dispersible, containing 97.86% of other ingredients, including an adjuvant that has methanol. DNA lesion-specific repair enzymes, namely formamidopyrimidine DNA glycosylase (FPG) and endonuclease III (EndoIII) were purchased from Professor Andrew Collins (University of Oslo, Norway). All the other chemicals needed to perform comet assay were obtained from the Sigma-Aldrich Chemical Company (Spain).

2.2 Test animals and experimental design

Red swamp crayfish (*Procambarus clarkii*) specimens with an average length of 12.5 ± 0.05 cm (adults) were captured from two different populations, with two different origins. One population (P1) was collected in the Minho river (Vila Nova de Cerveira, Portugal) and was designed as reference population, taking into consideration the information available in the literature that describes the location as free of contamination (IST/INAG, 2001; Santos et al., 2013), namely concerning pesticides. The other population (P2) originated from Salreu (between Antuã and Vouga rivers) was collected in the Ria de Aveiro surrounding area (Portugal). This location was chosen due to its contamination history concerning seasonal applications of pesticides, namely the herbicide Viper® (information obtained through local sources), being referred as historically impacted.

After captures, *P. clarkii* were acclimated in the laboratory for 15 days and kept in 60-L aquariums under a natural photoperiod, in aerated, filtered, dechlorinated and recirculating tap water, with the following physico-chemical conditions: salinity 0, temperature 19 ± 1°C, pH 7.1 ± 0.3, nitrate 27 ± 0.2 mg L^{-1}, nitrite 0.07 ± 0.02 mg L^{-1}, ammonia 0.2 ± 0.04 mg L^{-1}, dissolved oxygen 8.2 ± 0.3 mg L^{-1}. During this period, organisms were fed with squid *ad libitum*.

The experiment was carried out in 1-L aquaria, in a static mode, under the conditions previously described for the acclimation period. Crayfish specimens from each population (P1 and P2) were then divided in 4 groups, each with 12 individuals (6 males and 6 females, n=6), corresponding to a negative control with clean water (C), a model genotoxicant (MG) consisting in a solution of 5 mg L^{-1} of ethyl methanesulfonate (EMS) and two different concentrations of the herbicide Viper®, 20 µg L^{-1} (V1) and 40 µg L^{-1} (V2). EMS was chosen as positive control due to its well-known genotoxic properties (Cavas, 2011; Hartman et al., 2003). Viper® concentrations were chosen according to literature information which consider 20 µg L^{-1} (V1) as an environmental realistic concentration (Murussi et al., 2014; Rodrigues and Almeida, 2005) and 40 µg L^{-1} (V2) as the highest ecological estimated value, predicted by a model (EPA, 2004).

The rationale concerning the experimental design is depicted in Figure 3. Thus, organisms from both populations were exposed during 7 days to the 4 different conditions, mentioned above. During the experimental period, the water mediums were daily renewed and crayfish were not fed.

After the exposure, crayfish from P1 and P2 populations were sacrificed by a transection on the posterior side of the rostrum, followed by the removal of the carapace and the extraction of the gills onto a petri dish (one for each animal) with PBS (1.5 mL) to wash the tissue. Each gill was minced, and the up-and-down technique was performed in order to release as many cells as possible. Each animal' gills suspension was placed into a different microtube.

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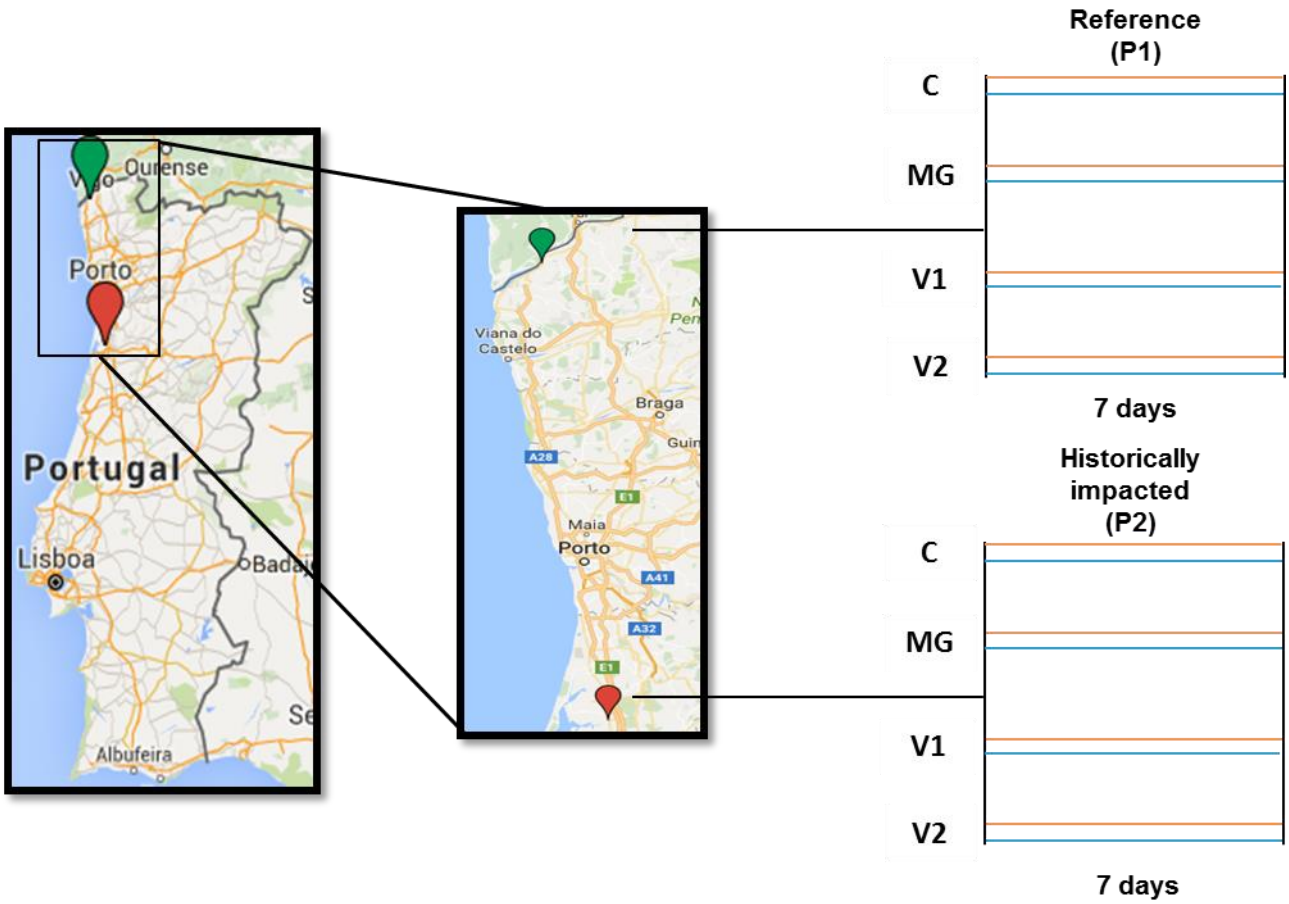

Figure 3. Schematic localization of collecting sites, where the reference population (P1) came from the Minho river (green) while the historically impacted population (P2) was collected in Salreu (red). The experiment rationale is also depicted, considering both populations (P1 and P2) exposure to four experimental conditions, with gender separation: males (blue) and females (orange). Images were adapted from Google Earth®.

2.3 Evaluation of genetic damage

2.3.1 Comet assay

The conventional alkaline version of the comet assay was performed according to the methodology of Collins (2004) as adapted by Guilherme et al. (2010), with proper adjustments to the procedure, including the extra step of digesting the nucleoids with endonucleases. A system of six gels per slide was adopted based on a model created by Guilherme et al. (2014a). Previously, 20 µL of cell suspension (previously prepared in PBS) was mixed with 70 µL of 1 % low melting point agarose (in PBS). Six drops of 6 µL were placed onto the precoated slide as two rows of 3 (3 groups of 2 replicates), without coverslips. The gels were left for

±5 min at 4 ºC in order to solidify the agarose, and then immersed in a lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, 1% Triton X-100, pH 10) at 4 ºC, for 1 h. After lysis of agarose-embedded cells, slides were washed 3 times with enzyme buffer (0.1 M KCl, 0.5 mM EDTA, 40 mM HEPES, 0.2 mg mL^{-1} bovine serum albumin, pH 8) at 4 ºC.

Three sets of slides were prepared: two sets were incubated with endonucleases FPG or EndoIII, which convert oxidized purines and pyrimidines into DNA single strand breaks, respectively (Azqueta et al., 2009), and a third set was incubated only with buffer. Hence, 30 µL of each enzyme (diluted in buffer) were applied in each gel, together with a coverslip, prior to incubation at 37ºC for 30 min in a humidified atmosphere. The slides were then gently placed in the electrophoresis tank, immersed in electrophoresis solution (15 min) for alkaline treatment. DNA was allowed to migrate at a fixed voltage of 25 V, a current of 300 mA which results in 1.04 V cm^{-1} (achieved by adjusting the buffer volume in the electrophoresis tank). The slides were stained with ethidium bromide (20 µg mL^{-1}).

Fifty nucleoids were observed per gel, using a Leica DMLS fluorescence microscope (400× magnification). The DNA damage was quantified by visual classification of nucleoids into five comet classes, according to the tail intensity and length, from 0 (no tail) to 4 (almost all DNA in tail) (Collins, 2004). The total score expressed as a genetic damage indicator (GDI) was calculated multiplying the percentage of nucleoids in each class by the corresponding factor, according to this formula:

GDI = [(%nucleoids class 0) x0] + [(%nucleoids class 1) x1] + [(%nucleoids class 2) x 2] + [(%nucleoids class 3) x 3] + [(%nucleoids class 4) x 4]

GDI values were expressed as arbitrary units in a scale of 0–400 per 100 scored nucleoids. When the comet assay was performed with additional FPG and EndoIII steps, GDI values were calculated in the same way and the parameters were designated GDI_{FPG} and $GDI_{EndoIII}$, respectively. More parameters were calculated based on the difference between $GDI_{EndoIII}$ and GDI, and GDI_{FPG} and

GDI, corresponding to additional DNA breaks, which are net enzyme-sensitive sites solely ($NSS_{EndoIII}$ and NSS_{FPG}, respectively).

2.4 Statistical analysis

Statistica 7.0 software company was used for statistical analysis. All data was first tested for normality and homogeneity of variances, and transformed in order to meet the required statistical demands.

A three-way ANOVA was used with the purpose of assessing the significant effects of population, treatment and gender factors, on assessed parameters, as well as their interaction. In order to identify significant differences within and between populations, treatments and genders, this analysis was followed by a post-hoc Tukey test. Differences between means were considered significant when $p<0.05$ (Zar, 1996).

3. Results

3.1 Non-specific DNA damage

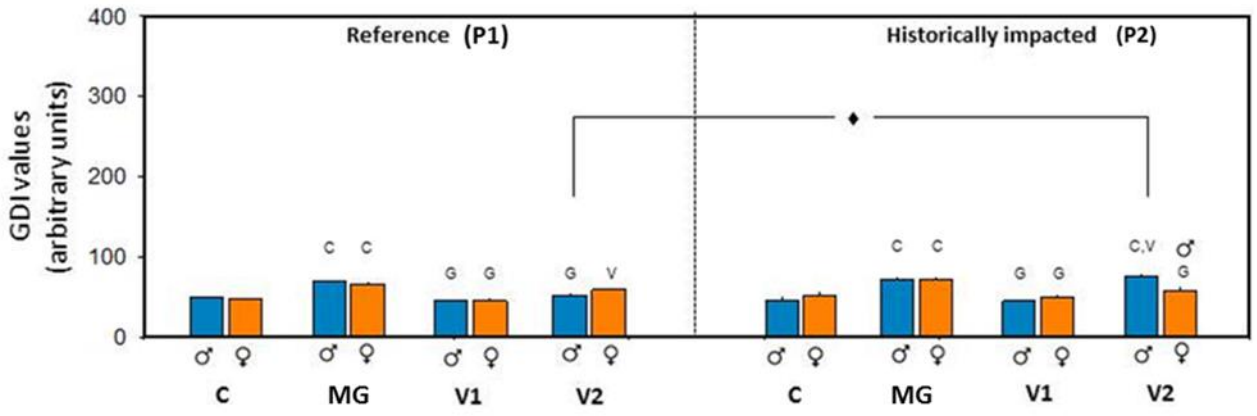

Figure 4. Mean values of genetic damage indicator (GDI) measured by the standard (alkaline) comet assay in adult gill cells of *P. clarkii* with two different origins, collected from a reference site (P1) and an historically impacted site (P2), and exposed during 7 days to laboratory different conditions: control (C), model genotoxicant (MG), Viper® 20 µg L^{-1} (V1) and Viper® 40 µg L^{-1} (V2). Males (♂) are represented in blue and females (♀) in orange. Bars represent the standard error. Statistically significant differences ($p<0.05$) are: in relation to control (C), in relation to model genotoxicant (G) and in relation to V1 (V), within the same population and gender; (♦) between sites, within the same treatment and gender; (♂) between genders, within the same population and treatment.

The analysis of GDI values (Figure 4), considering the reference population (P1), showed that both genders displayed a damage increase considering MG groups, when compared to respective controls (C). Males and females also presented a significant decrease in V1 groups in relation to MG. Moreover, and when the highest concentrations of Viper® (V2) were considered, males displayed significantly low damage levels than the same gender from MG group, while V2 females showed to be more affected when compared with females from the V1. The historically impacted population (P2) (including both genders) showed a similar pattern concerning MG and V1 groups (Figure 4). The main differences were related to the results presented by V2 groups, where males showed higher damage levels than in control and V1 treatments, and females presented a significant decrease in DNA damage when compared to MG. Additionally, a

difference between genders was observed, considering the highest concentration of Viper® (V2), where males displayed higher non-specific DNA damage than females. In what concerns to the comparison between populations, only an effective difference was observed and it was displayed by males from V2 groups (Figure 4).

Table 1. Summary of the three-way ANOVA (Univariate Tests of Significance) relative to the genetic damage indicator (GDI) measured in male and female gill cells of *P.clarkii*, with two different origins and submitted to different laboratory treatments. Significant values are signalized with an asterisk (*****).

Effect	df	MS	F	p
Population	1	495.0	8.490	**0.004629**
Treatment	3	2564.7	43.984	**0.000000**
Gender	1	11.3	0.195	0.660351
Population **x** Treatment	3	98.0	1.680	0.177944
Population **x** Gender	1	23.0	0.395	0.531667
Treatment **x** Gender	3	68.5	1.175	0.324619
Population **x** Treatment **x** Gender	3	360.4	6.180	**0.000785**
Residual	80	58.3		

Results depicted in Table 1 revealed a statistically significant interaction between the three considered factors (Population x Treatment x Gender) (Table 1).

3.2 Specific oxidative DNA damage

The detection of oxidized bases was achieved by the comet assay with an extra step where nucleoids were incubated with the DNA lesion-specific repair enzymes FPG and EndoIII (Figures 5 and 6).

3.2.1 FPG associated damage

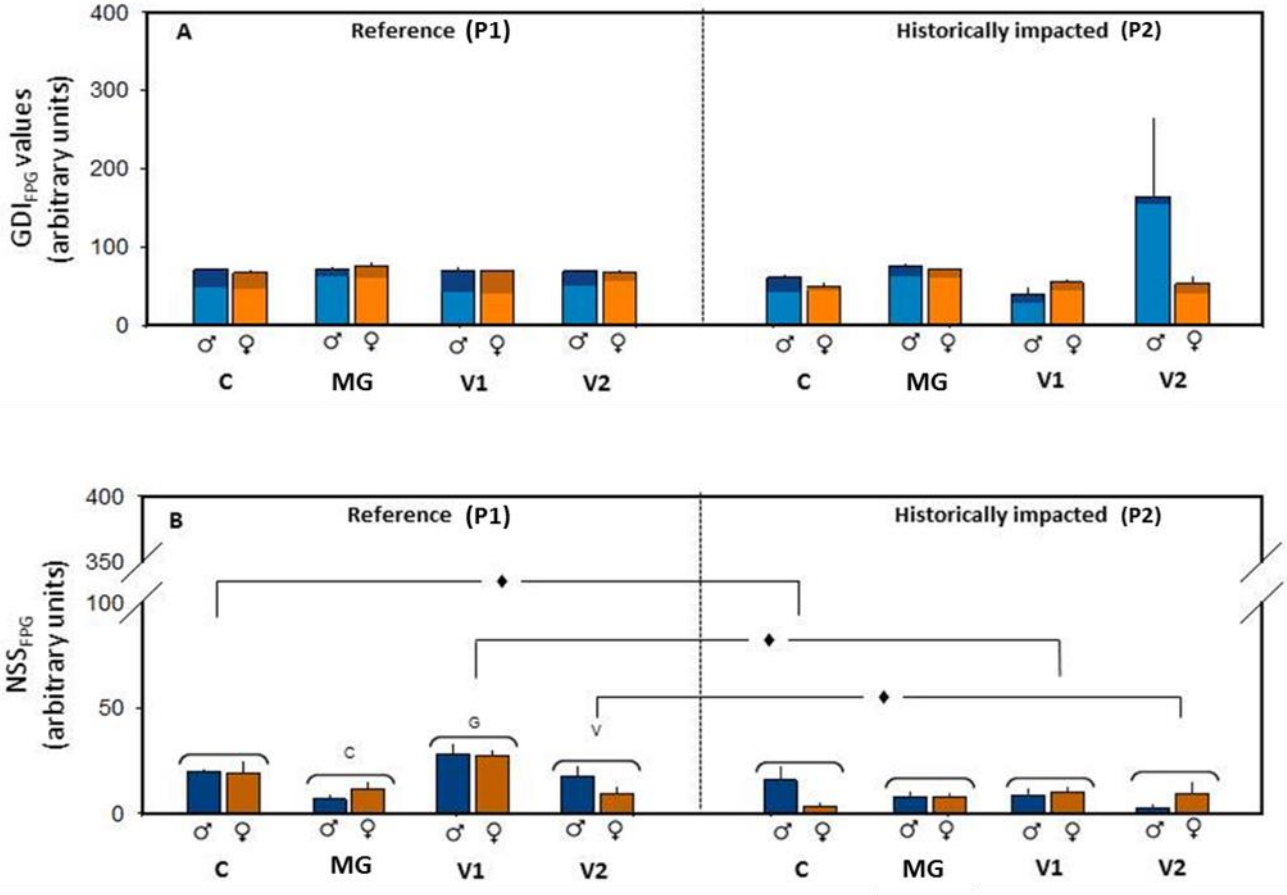

Figure 5. Mean values of genetic damage indicator (GDI) measured by the standard (alkaline) comet assay in adult gill cells of *P. clarkii* with two different origins, collected from a reference site (P1) and from an historically impacted site (P2), and exposed during 7 days to laboratory different conditions: control (C), model genotoxicant (MG), Viper® 20 µg L^{-1} (V1) and Viper® 40 µg L^{-1} (V2). Males (♂) are represented in blue and females (♀) in orange. Bars represent the standard error. Values resulted from the assay with an extra step of digestion with formamidopyrimidine DNA glycosylase (FPG) to detect oxidised purine bases: (A) overall damage (GDI_{FPG}) and partial scores, namely genetic damage indicator (GDI, light colour) and additional DNA breaks corresponding to net FPG sensitive sites (NSS_{FPG}, dark colour); (B) NSS_{FPG} alone. Due to the absence of differences between genders, the analysis of the NSS_{FPG} parameter (exclusively) was performed neglecting this factor. Statistically significant differences ($p<0.05$) are: in relation to control (C), in relation to model genotoxicant (G) and in relation to V1 (V), within the same population and gender; (♦) between sites, within the same treatment and gender.

The GDI_{FPG} parameter (Figure 5A) displayed no differences between groups, either considering P1 or P2. Also, no significant differences were noticeable neither between populations nor genders (Figure 5A).

On the other hand, the NSS_{FPG} parameter (Figure 5B), considering both genders together (since they presented similar results, according to the ANOVA three-way results), showed in P1 a significant decrease of MG group values, when compared to control. Additionally, the V1 group presented significantly higher values in relation to MG while the group corresponding to the highest concentration of Viper® (V2) displayed a significant decrease when compared to the lowest concentration (V1). In what concerns P2, no differences were found between treated groups (Figure 5B). When both populations were compared, significant differences were noticeable between C, V1 and V2 homologous groups (Figure 5B).

Table 2. Summary of the three-way ANOVA (Univariate Tests of Significance) considering (A) the genetic damage indicator with an extra step of digestion with formamidopyrimidine DNA glycosylase (GDI_{FPG}) to detect oxidised purine bases and (B) DNA breaks corresponding to net FPG sensitive sites (NSS_{FPG}). Significant values are signalized with an asterisk (*****).

A

Effect	df	MS	F	p
Population	1	495.0	8.490	0.004629
Treatment	3	2564.7	43.984	0.000000
Gender	1	11.3	0.195	0.660351
Population **x** Treatment	3	98.0	1.680	0.177944
Population **x** Gender	1	23.0	0.395	0.531667
Treatment **x** Gender	3	68.5	1.175	0.324619
Population **x** Treatment **x** Gender	3	360.4	6.180	0.000785
Residual	80	58.3		

B

Effect	df	MS	F	p
Population	1	2076.69	25.5065	**0.000003**
Treatment	3	454.22	5.5788	**0.001585**
Gender	1	38.13	0.4683	0.495751
Population **x** Treatment	3	277.01	3.4023	**0.021635**
Population **x** Gender	1	0.07	0.0008	0.977511
Treatment **x** Gender	3	84.21	1.0343	0.381997
Population **x** Treatment **x** Gender	3	194.40	2.3877	0.075069
Residual	80	81.42		

The analysis of the three-way ANOVA outputs, respecting to the GDI_{FPG} parameter, didn't displayed any significances (Table 2A).

On the other hand, results related to NSS_{FPG} parameter strengthened the detected differences between populations and treatments since it was possible to observe a statistically significant interaction between these factors (Table 2B)

3.2.2 EndoIII associated damage

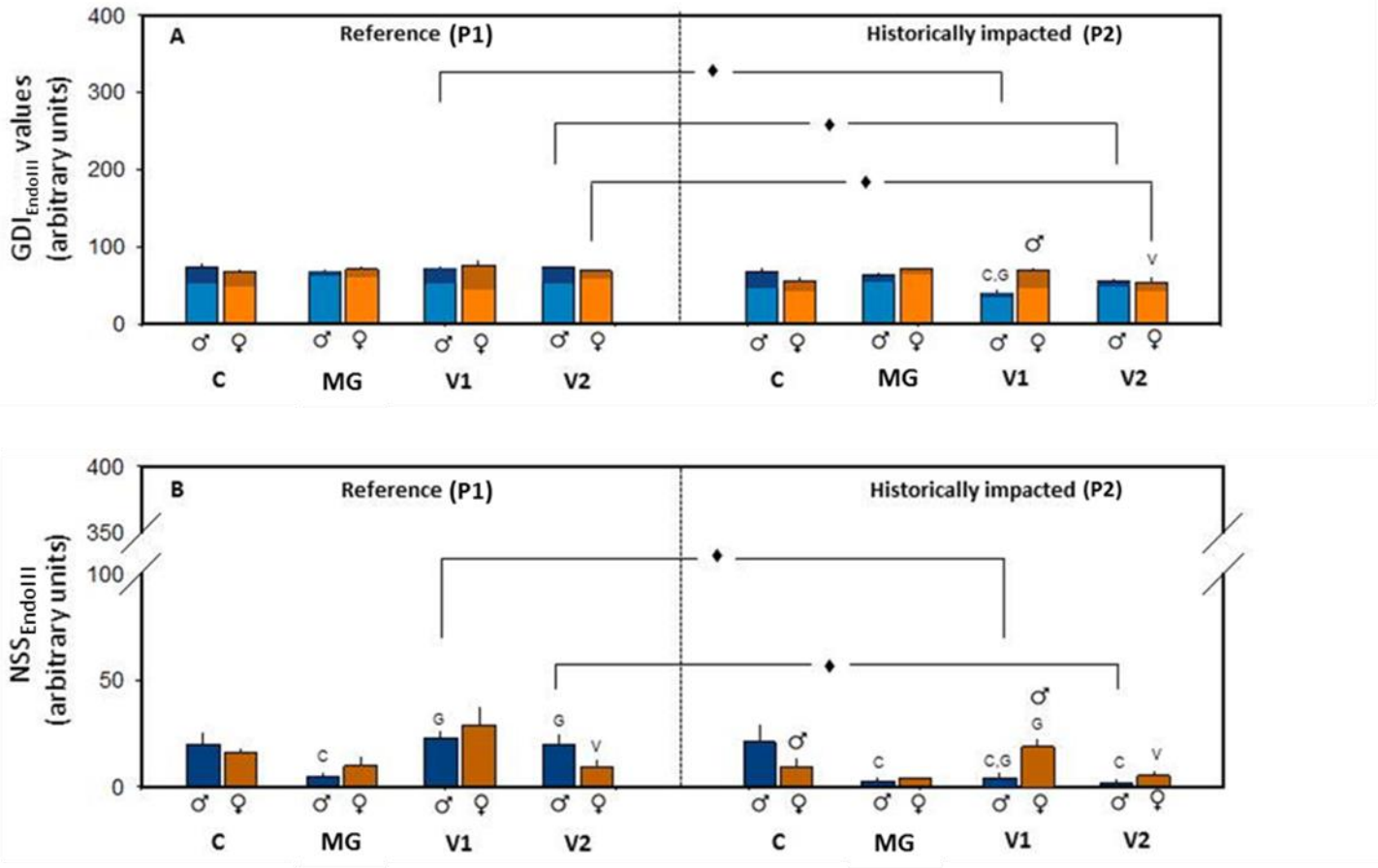

Figure 6. Mean values of genetic damage indicator (GDI) measured by the standard (alkaline) comet assay in adult gill cells of *P. clarkii* with two different origins, collected from a reference site (P1) and from an historically impacted site (P2), and exposed during 7 days to laboratory different conditions: control (C), model genotoxicant (MG), Viper® 20 µg L^{-1} (V1) and Viper® 40 µg L^{-1} (V2). Males (♂) are represented in blue and females (♀) in orange. Bars represent the standard error. Values resulted from the assay with an extra step of digestion with endonuclease III (EndoIII) to detect oxidised pyrimidine bases: (A) overall damage ($GDI_{EndoIII}$) and partial scores, namely genetic damage indicator (GDI, light colour) and additional DNA breaks corresponding to net EndoIII sensitive sites ($NSS_{EndoIII}$, dark colour); (B) $NSS_{EndoIII}$ alone. Statistically significant differences ($p<0.05$) are: in relation to control (C), in relation to model genotoxicant (G) and in relation to V1 (V), within the same population and gender; (♦) between sites, within the same treatment and gender and (♂) between genders, within the same population and treatment.

The analysis of $GDI_{EndoIII}$ parameter (Figure 6A), in what concerns P1, didn't displayed any differences between treated groups. On the other hand, P2 (Figure 6A) presented significant decreases in V1 group, in relation to C and MG groups, when males were considered. Moreover, V2 females were less sensitive to DNA damage than V1 ones. Additionally, a difference between genders was observed considering the lowest concentration of Viper® (V1), where males displayed a lower specific DNA damage than females. In what concerns to the comparison between populations, differences were displayed by males concerning V1 and V2 groups and females corresponding to V2 group (Figure 6A).

The examination of the $NSS_{EndoIII}$ parameter (Figure 6B), regarding P1, noticed that males from the MG group showed a damage decrease comparatively to control (C). Males from both Viper® concentrations (V1 and V2) displayed a damage increase comparatively to males of MG group. Also, females from V2 group presented lower damage levels when compared with the same gender in V1. Observing P2 (Figure 6B), it was noticed that males from MG, V1 and V2 groups showed significant decreases when compared with C group. In the other way, males from V1 group presented higher DNA damage values than MG males. Moreover, females belonging to the V1 group showed higher values when compared with their homologous from MG, while V2 females presented lower damage levels when compared with V1 ones. Additionally, two differences between genders were observed, namely in the control group (C), where males displayed higher specific DNA damage than females, and V1 where the opposite pattern occurs. Concerning the comparison between populations (Figure 6B), differences between males were observed concerning V1 and V2 groups.

Table 3. Summary of the three-way ANOVA (Univariate Tests of Significance) considering (A) the genetic damage indicator with an extra step of digestion with endonuclease III (EndoIII) to detect oxidised pyrimidine bases and (B) DNA breaks corresponding to net EndoIII sensitive sites ($NSS_{EndoIII}$). Significant values are signalized with an asterisk (*****).

A

Effect	df	MS	F	p
Population	1	3510.2	33.689	**0.000000**
Treatment	3	148.8	1.428	0.240680
Gender	1	99.0	0.950	0.332561
Population **x** Treatment	3	226.8	2.177	0.097163
Population **x** Gender	1	129.5	1.243	0.268249
Treatment **x** Gender	3	703.0	6.747	**0.000409**
Population **x** Treatment **x** Gender	3	269.6	2.588	0.058734
Residual	80	104.2		

B

Effect	df	MS	F	p
Population	1	30.1022	14.5196	**0.000271**
Treatment	3	19.0199	9.1741	**0.000028**
Gender	1	0.0481	0.0232	0.879358
Population **x** Treatment	3	7.9514	3.8353	**0.012762**
Population **x** Gender	1	0.3952	0.1906	0.663577
Treatment **x** Gender	3	8.4677	4.0844	**0.009435**
Population **x** Treatment **x** Gender	3	11.6074	5.5988	**0.001548**
Residual	80	2.0732		

The analysis of the $GDI_{EndoIII}$ parameter, present in Table 3A, pointed a significant interaction between factors treatment and gender (Table 3A).

In what concerns to the $NSS_{EndoIII}$ parameter, the influence of factors as population and treatment was evidenced (Table 3B) since it was also possible to observe statistically significant interactions between the considered factors, namely population x treatment, treatment x gender and population x treatment x gender (Table 3B).

4. Discussion

The genotoxicity of pesticides to aquatic organisms has been widely investigated. Several studies demonstrated their ability to induce genetic damage, with different magnitudes and considering various endpoints (Ansoar-Rodríguez et al., 2015; Guilherme et al., 2014a; Marques et al., 2016, 2014a, 2014b). Thus, the present study intended to assess the genotoxicity of the commercial herbicide Viper®, since it remains unknown, but also to shed a light on potential adjustment strategies developed by well-succeeded species, in order to cope with pesticide environmental contamination. In addition, a well-known model genotoxicant (EMS) was also tested, in order to assess whether adjustment strategies eventually developed can have repercussions extensible to genotoxic responses to a novel (absent in the exposure history) DNA challenge. Bearing all these in mind, adults of the crayfish *Procambarus clarkii*, belonging to two distinct populations (with different contamination backgrounds) were exposed to environmentally relevant concentrations of the mentioned chemicals. Moreover, and to complement the approach, males and females were tested in separate to appraise the influence of the factor gender.

4.1 Genotoxicity responses and their determining factors

4.1.1 Non-specific DNA damage

In what concerns to the non-specific DNA damage (GDI parameter), the herbicide Viper® expressed its ability to exert genotoxicity only considering the males group that was exposed to the highest concentration of Viper®, from the historically impacted population (P2). This fact points out gender-specific responsiveness, expressed as a higher sensitivity of males to the herbicide, namely after a pre-exposure (yet in the field), which is corroborated by the gender-associated difference between males and females from V2 group. As a consequence of these responses, a difference between the tested populations

was found, when males were considered, pointing out a higher susceptibility of P2 in relation to non-specific DNA damage.

In what concerns to the model genotoxicant (MG group), both populations presented a similar response showing its ability in exert non-specific DNA damage, regardless the populations' exposure historical and crayfish gender. Despite this, differences between populations were reinforced by the significant influence of the factor "Population", which showed also to interact with the other two factors ("Treatment" and "Gender").

4.1.2 Oxidative DNA damage

As an attempt to assess the eventual involvement of oxidative damage on the DNA integrity loss, the DNA lesion-specific repair enzymes were included to signalize oxidised purines and pyrimidines (FPG and EndoIII, respectively). Consequently, different patterns were observed when these parameters were assessed. The GDI_{FPG} parameter, beyond not displaying neither the Viper® nor the EMS genotoxicity, didn't present differences between populations, fact that was accompanied by the absence of factors' significant influence and their interaction, considering the holistic statistical analysis. In a different way, the NSS_{FPG} parameter revealed a new perspective. The statistical analysis, contrary to all the other assessed parameters, dictated that gender responses appeared as similar, resulting thus into a unique group per treatment. Other interesting information was that the model genotoxicant (EMS), in P1 (Reference population), presented a significant NSS_{FPG} decrease when compared to control and, at the same time, also when compared to V1 group. Since the baseline values of the control group were considered low, namely when compared to *Anguilla anguilla* L. for example (Guilherme et al., 2012; Guilherme et al., 2014a; Marques et al., 2014a), this fact may indicate that the group treated with EMS (MG group) was able to activate cell defence mechanisms of DNA repair and/or antioxidant system, as already stated by Marques et al (2014b), resulting thus in extremely low values of oxidative damage. This tendency was also perceptible in P2, despite the absence of statistical significances. Notwithstanding the lack of evidences concerning Viper®

ability to induce oxidative stress, in disagreement with the toxic effects demonstrated in daphnia (Marques et al., 2012, Suárez-Serrano et al., 2010b), in P2 all the treatments, with the exception to MG group, presented lower damage values (including the control group) in relation to P1, indicating probably the occurrence of an adjustment strategy not transversal to all kinds of genotoxic pressure. These facts point to an improvement of this population defences (P2), probably associated to the pre-exposure to pesticides, allowing to deal better with environmental stressors, namely in what concerns to the tested herbicide. The differences between populations, considering responses to the same stimulus, are also supported by the significant influence of factors “Population” and “Treatment”, as well as their interaction.

EndoIII-associated DNA breaks are recognized to reflect the presence of oxidised pyrimidines. These enzyme-related parameters must be analysed jointly with those relative to FPG, since they frequently present different response patterns (Guilherme et al., 2014b; Marques et al., 2014b). Considering this, the overall damage ($GDI_{EndoIII}$) reflected differences between populations, which are reinforced by the factor “Population” influence itself. This parameter also highlighted males’ distinct responses (as well as differences between populations) since only V1 males from P2 presented a significant decrease in DNA damage values, in comparison to C group, revealing an improvement in the defences against oxidative DNA damage. This finding is also reinforced by V1 and V2 males’ (and females corresponding to V2 group) distinct responses between P1 and P2. Thus, this parameter also supports the theory of an adjustment strategy developed by P2 organisms, reflecting a better strategy to cope with the herbicide exposure. The difference between genders (once again considering the historically impacted population) was also present in V1 group, where females presented higher damage levels, demonstrating their lower ability to deal with DNA injuries. Still considering females from P2, the comparison between both Viper® groups showed that organisms exposed to the lowest concentration appeared to be more affected than the others, highlighting the occurrence of different responses depending on the herbicide concentration. Differential female’ responses in relation to males, considering Viper® groups, were reinforced by the existent

interaction between factors “Treatment” and “Gender”, revealing thus different gender abilities to deal with genotoxic stressors. The evaluation of DNA breaks relative to oxidised pyrimidines ($NSS_{EndoIII}$ parameter) revealed, once again, population-specific patterns. The reference population (P1) demonstrated that Viper® was not able to exert oxidative DNA damage. However, males from the MG group presented values below the control levels. This fact may be explained considering the oxidant potential of EMS (Ansari et al., 2011) that unleashed either the antioxidant defences and/or the DNA repair machinery.

The analysis of the historically impacted population (P2) draw attention, once more, to the development of adjustment strategies, curiously just considering males. In this gender, either MG or both Viper® treatments displayed significant decreases of oxidised pyrimidines when compared with control, revealing that a pre-exposure to Viper® (and eventually to other pesticides, since this population have been exposed during its lifetime) promoted an additional protection to crayfish in what concerns to the oxidative DNA damage. It can be stated that these crayfish, mainly males, are equipped with extra defences (either antioxidant or DNA repair mechanisms) that provided a prompt response to the oxidative challenge posed by Viper®. This adjustment acquisition was reinforced by differences found between males exposed to Viper® from the two populations (P1 *vs*. P2), as well as by the influence of the factor “Population” pointed by statistics and also by the significant difference between V1 genders (in P2). In what concerns EMS, the significant decrease of oxidised pyrimidines observed in males when compared with control in P2 cannot be interpreted as a consequence from an adjustment process associated to the exposure historic, since the same response profile was observed in P1. Hence, it is reinforced the idea of a specificity of the perceived adjustment processes in what concerns the type of DNA insulting agent. Despite the absence of significance in relation to control, females don’t appear to be equally protected, since their defences don’t seem to be induced. Moreover, V2 females presented a significant decrease when compared to V1 (in both populations), pointing a differential response related to the magnitude of the stimulus (herbicide concentration) and also reinforcing the idea that only males present different response profiles between populations.

Overall, the $NSS_{EndoIII}$ parameter demonstrated to be the most affected by the influence of the factors under consideration ("Population", "Treatment" and "Gender"), as well as their interactions. Responses given by this parameter strongly reflect the complexity of factors that may influence organism's genotoxic responses.

The present approach highlighted the importance of a holistic evaluation considering genotoxicity parameters. Several published studies enhanced the comet assay as an excellent tool to assess DNA breaks, namely induced by environmental genotoxicants (Cotelle and Ferard, 1999; Jha, 2008; Mitchelmore and Chipman, 1998). However, in general, approaches in the literature only consider the evaluation of the non-specific damage, which reveals a broad spectrum of recent lesions that are susceptible of being repaired, as well as DNA strand-breaks and alkali labile sites (Andrade et al., 2004; Lee and Steinert, 2003; Speit and Schütz 2008). In order to shed light on the eventual oxidative cause in the observed damage, our approach included an extra-step in the protocol where bacterial repair endonucleases detect oxidised bases (Collins, 2004). Therefore, the use of formamidopyrimidine DNA glycosylase (FPG) and Endonuclease III (EndoIII) had highly increased the sensitivity of the assay, allowing thus the detection of specific damage, namely the oxidative (Azqueta et al., 2009). These ideas were also mentioned in our team studies (Guilherme et al., 2015, 2014a, 2014b, 2012; Marques et al., 2014b), which highlighted the differential patterns depicted by the selected parameters. Considering the non-specific damage evaluation (GDI) in particular, the current study revealed that both populations react almost in the same way to the challenges, with the exception of V2 males that proved the genotoxicity of Viper®. However, when we take a look to the "complementary parameters", *viz*. GDI_{FPG}, NSS_{FPG}, $GDI_{EndoIII}$ and $NSS_{EndoIII}$, other perspective was apparent. In particular, NSS_{FPG} and $NSS_{EndoIII}$ parameters revealed substantially different responses between populations and these results are not consistent with those signalized by standard comet assay (GDI) data, pointing thus a limitation of the standard methodology, as already stated by Guilherme et al. (2012). Moreover, it is currently accepted that the use of the DNA lesion-specific repair enzyme FPG may be sufficient to point the occurrence of

oxidative damage (Gielazyn et al., 2003; Speit et al., 2004). The present work appears as an excellent example to contest this assumption, based on the fact that it only signalizes oxidized purines while pyrimidines oxidation is automatically excluded. In this direction, only the $NSS_{EndoIII}$ parameter allowed to perceive the consistent outcome concerning the males triggering of the protection mechanism, either for the herbicide or the model genotoxicant exposure.

4.2 Contribution for the knowledge about mechanisms involved in positive adjusts or vulnerabilities of crayfish

Throughout this discussion, it has been mentioned that some organisms somehow reflected an adjustment phenomenon conditioned by their origin. This rationale was based in the assumption that populations that have already been exposed to pesticides are able to better deal with a future challenge, namely concerning the same pollutant. As hypothesised, current outcomes confirmed this ability. Briefly, male crayfish from P2 (population historically exposed to Viper®), showed to be able to deal with the damage inflicted by Viper® while this skill was not extensible to the model genotoxicant, since MG groups presented a similar pattern concerning both populations. However, it is possible to state that P2 males appear to be better equipped than females in what concerns to avoid oxidative DNA damage.

On the other hand, despite these favourable points, there are also some disadvantages to signalize. Contrary of what happens with the protection against the oxidative DNA damage, male crayfish from P2 were not able to deal with non-specific damage, as demonstrated by GDI parameter. Moreover, other conditionings must also be considered. The energy allocation relative to the defences' induction might have a negative impact in the organism fitness and performance. So, a question may arise: will be the final balance between advantages and disadvantages positive for individuals? Since survival is the ultimate advantage that a living being can accomplish, the final balance seems to be extremely positive, since *Procambarus clarkii* is considered as a well-succeeded species around the world.

4.2.1 Tolerance *vs*. resistance

As mentioned in the introduction section, tolerance and resistance are different concepts, reflecting differences on the underlying processes. While the former reflects a physiological adjustment that comes from a direct exposure of the organism to a genotoxicant agent, the latter considers the existence of a genetic adaptation which is inheritable by the progenitors. In what concerns the present study, tested adults may reflect both phenomena. If on one hand, they may have inherited some genetic information from their ancestors, on the other, they have also been exposed during their lifetime to pesticides, which may provide, *per se*, an acquisition of tolerance to genotoxicants. Since it was not possible to understand which adjustment mechanism was reflected by these organisms, perhaps the use of other life stadium, as juveniles which have never been exposed, could help with this clarification.

5. Conclusions

The genotoxicity of the herbicide Viper® to the non-target species *Procambarus clarkii* (crayfish) was proved, despite only evidenced in males from the historically impacted population. The inclusion of a model genotoxicant (EMS) proved to be essential since it allowed in perceiving the acquisition of a non-specific protection/vulnerability against genetic damage. In this direction, the impact of the exposure history showed to depend on the damaging event under analysis, on the challenging agent at play, as well as on the gender. Hence, organisms from the historically impacted population revealed a higher susceptibly towards the non-specific genotoxic pressure (GDI) posed by Viper®, while in relation to DNA oxidation (mainly as pyrimidine lesions) the same population showed an increased ability to deal with this type of damage. Moreover, these repercussions of the exposure history were only evident in males and in relation to the agent Viper®, thereby not extensible to EMS, an agent absent in the past of crayfish.

Therefore, considering the genotoxic evaluation as a whole, the influence of factors as “Population” and “Gender” was demonstrated, highlighting the importance of consider differences on the organisms’ physiological background for ecogenotoxicological-based environmental health assessment, permitting the elaboration of more plausible and holistic approaches.

6. References

Abdel-kader, S. M. (2016). The Egyptian German Society for Zoology Some ecological and toxicological studies on crayfish, *Procambarus clarkii* at Sharkia. The Journal of Basic & Applied Zoology, *74*, 62–67.

Akcha, F., Leday, G., and Pfohl-leszkowicz, A. (2004). Measurement of DNA adducts and strand breaks in dab (*Limanda limanda*) collected in the field: effects of biotic (age, sex) and abiotic (sampling site and period) factors on the extent of DNA damage. Mutation Research, *552*, 197–207.

Aktar, M. W., Sengupta, D., and Chowdhury, A. (2009). Impact of pesticides use in agriculture: their benefits and hazards. Interdisciplinary Toxicology, *2* (1), 1–12.

Anderson, M.B., Preslan, J.E., Jolibois, L., Bollinger, T.E., George, W.J. (1997). Bioaccumulation of lead nitrate in red swamp crayfish (*Procambarus clarkii*). Journalof Hazardous Materials, 54, 15–29.

Andrade, VM., Silva, J., Silva, FR., Heuser, VD., Dias, JF., Yoneama, ML. (2004) Fish as bioindicators to assess the effects of pollution in two southern Brazilian rivers using the Comet assay and micronucleus test. Environmental and Molecular Mutagenesis, 44:459–468.

Ansari, R.A., Rahman, S., Kaur, M., Anjum, S., Raisuddin, S. (2011). In vivo cytogenetic and oxidative stress-inducing effects of cypermethrin in freshwater fish, *Channa punctata* Bloch. Ecotoxicology and Environmental Safety, 74, 150–156.

Ansoar-Rodríguez, Y., Christofoletti, C., Marcato, A., Correia, J., Bueno, O., Malaspina, O., and Fontanetti, C. (2015) Genotoxic Potential of the Insecticide Imidacloprid in a Non-Target Organism (*Oreochromis niloticus*-Pisces). Journal of Environmental Protection, 6, 1360-1367.

Azqueta, A., Shaposhnikov, S., Collins, A., (2009). DNA oxidation: investigating its key role in environmental mutagenesis with the comet assay. Mutation Research, 674, 101–108.

Belenguer, V., Martinez-Capel, F., Masiá, A., Picó, Y. (2014). Patterns of presence and concentration of pesticides in fish and waters of the Júcar River (Eastern Spain). Journal of Hazardous Materials, 265: 271–9.

Benli, A.C.K., Sarikaya, R., Sepici-Dincel, A., Selvi, M., Sahin, D., Erkoç, F. (2007). Investigation of acute toxicity of (2,4-dichlorophenoxy) acetic acid (2,4-D) herbicide on crayfish (Astacus leptodactylus Esch. 1823). Pesticide Biochemistry and Physiology, 88, 296–299.

Bleich, O. (2006). Exposure of population to genotoxic factors from the environment. Univerzita Karlova v Praze.

Bickham, J. W. (2000). Effects of chemical contaminants on genetic diversity in natural populations : implications for biomonitoring and ecotoxicology. Mutation research, 463, 33-51.

Biggs, J., Williams, P., Whitfield, M., Nicolet, P., Brown, C., Hollis, J.,Pepper, T. (2007). The freshwater biota of British agricultural landscapes and their sensitivity to pesticides. Agriculture, Ecosystems and Environment, 122 (2), 137–148.

Bolognesi, C. (2003). Genotoxicity of pesticides: a review of human biomonitoring studies. Mutation Research/Reviews in Mutation Research, 543 (3): 251–272.

Boxall, ABA., Brown, CD., Barrett, KL. (2002). Review - Higher-tier laboratory methods for assessing the aquatic toxicity of pesticides. Pest Management Science, 58, 637-648.

Brausch, J. M., and Smith, P. N. (2009). Pesticide resistance from historical agricultural chemical exposure in *Thamnocephalus platyurus* (Crustacea: Anostraca). Environmental Pollution, 157 (2), 481–487.

Bruno, JF., Bertness, MD. (2001). Habitat modification and facilitation in benthic marine communities. In: Bertness MD, Gaines SD, Hay ME, editors.

Marine community ecology. Sunder- land, MA: Sinauer Associates, Inc,. p. 201–218.

Carrasco, K., Tilbury, K., Myers, M. (1990). Assessment of the piscine micronuclei test as an in situ biological indicator of chemical contaminant effects. Canadian Journal of Fisheries and Aquatic Sciences, 47,2123-2136.

Cavas, T. (2011). In vivo genotoxicity evaluation of atrazine and atrazine-based herbicide on fish *Carassius auratus* using the micronucleus test and the comet assay. Food and Chemical Toxicology : An international journal published for the British Industrial Biological Research Association, 49 (6), 1431–5.

Cotelle, S., and Ferard, J. F. (1999). Review Comet Assay in Genetic Ecotoxicology : A Review. Environmental and Molecular Mutagenesis, *255*(February), 246–255.

Collins, A. R. (2004). "The comet assay for DNA damage and repair: principles, applications, and limitations". Molecular Biotechnology, 26 (3), 249–61.

Dabrowski, JM., Bollen, A., Schulz, R.M. (2005). Combined effects of discharge, turbidity, and pesticides on mayfly behavior: experimental evaluation of spray-drift and runoff scenarios. Environmental Toxicology and Chemistry, 24(6), 1395-1402.

Dawes, A.J. (1981). The Louisiana Red swamp crayfish (*Procambarus clarkii*) - a crustacean suitable for classroom use. Journal of Biological Education, 15 (1), 25-30.

De la Sienra, E., Armienta, M., Gonsebatt, M. (2003). Potassium dichromate increases the micronucleus frequency in the crayfish *Procambarus clarkii*. Environmental Pollution, 126 (3), 367–370.

Depledge, M. H. (1994). Genotypic toxicity: Implications for individuals and populations. Environmental Health Perspectives, 102 (SUPPL. 12), 101–104.

Desouky, M.M., Abdel-Gawad, H., Hegazi, B. (2013). Distribution, fate and histo- pathological effects of ethion insecticide on selected organs of the crayfish, *Procambarus clarkii*. Food and Chemical Toxicology, 52, 42–52.

Diekmann, M., Hultsch, V., and Nagel, R. (2004). On the relevance of genotoxicity for fish populations I: Effects of a model genotoxicant on zebrafish (*Danio rerio*) in a complete life-cycle test. Aquatic Toxicology, *68*(1), 13–26.

Dow AgroSciences. (2002). Research Sample Safety Data Sheet – Product: GF-1076. Dow AgroSciences LLC, Indianapolis.

Dronkert, ML., Beverloo, HB., Johnson, RD., Hoeijmakers, JH., Jasin, M., Kanaar, R.Mouse. (2000). RAD54 affects DNA double-strand break repair and sister chromatid exchange. Molecular and Cellular Biology, 20 (9), 3147- 3156.

Dudaš A., Chovanec M. (2003). Review: DNA double-strand break repair by homologous recombination. Mutation Research, 566, 131-16.

EPA. (2004). Pesticide Fact Sheet for Penoxsulam www.epa.gov/opprd001/factsheets/penoxsulam.pdf.

Essers, J., van Steeg, H., De Wit, J., Swagemakers, SM., Vermeij, M., Hoeijmakers JH., Kanaar R. (2000). Homologous and non-homologous recombination differentially affect DNA Damage repair in mice. EMBO Journal , 19, 1703-1710.

Fenech, M. (2000). The in vitro micronucleus technique. Mutation Research, 455,81-95.

Fleeger, J. W., Carman, K. R., and Nisbet, R. M., (2003). Indirect effects of contaminants in aquatic ecosystems. Science of the Total Environment, *317* (1-3), 207–233.

Forbes, V. E. (2000). Is hormesis an evolutionary expectation? Functional Ecology, 14 (1), 12–24.

Fortoul, T. I., Valverde, M., Lopez, M. C., Avila-Costa, M. R., Avila-Casado, M. C., Mussali-Galante, P.,Ostrosky-Shejet, P. (2004). Genotoxic differences by sex in nasal epithelium and blood leukocytes in subjects residing in a highly polluted area. Environmental Research Journal, *94* (3), 243–248.

Frenzilli, G., Nigro, M., Lyons, B.P, (2009). The comet assay for evaluation of genotoxic impact in aquatic environments. Mutation Research, 681, 80–92.

Gavio, M.A., Orensanz, J.M., Armstrong, D. (2006). Evaluation of alternative life history hypotheses for the sand shrimp *Crangon franciscorum* (Decapoda: Caridea). Journal of Crustacean. Biology, 26, 295–307.

Gaudé, A.P. (1984). Ecology and production of Louisiana red swamp crayfish *Procambarus clarkii* in Southern Spain. In: Freshwater crayfish VI. Papers from the sixth international symposium on freshwater crayfish, Per Brinck, ed., International Assotiation of Astacology, 111-130.

Gherardi, F.,S., Barbaresiand, G.Salvi. (2000).Spatialandtemporal patterns in the movement of *Procambarus clarkii*, an invasive crayfish. Aquatic Sciences, 62, 179–193.

Gherardi, F., S. Barbaresi, O., Vaselli and A., Bencini. (2002). A comparison of trace metal accumulation in indigenous and alien freshwater macro-decapods. Marine and Freshwater Behaviour and Physiology, 35, 179–188.

Gherardi F., Lazzara, L. (2006). Effects of the density of an invasive crayfish (*Procambarus clarkii*) on pelagic and surface microalgae in a Mediterranean wetland. Archiv fur Hydrobiologie, 165, 401–414.

Gielazyn, M.L., Ringwood, A.H., Piegorsch, W.W., Stancyk, S.E., (2003). Detection of oxidative DNA damage in isolated marine bivalve hemocytes using the comet assay and formamidopyrimidine glycosylase (FPG). Genetic Toxicology and Environmental Research, 542, 15–22.

Gonzalez, F.J., Nebert, D.W. (1996). Evolution of the P450 superfamily: animal–plant ‘‘warfare’’, molecular drive, and human genetic differences in drug oxidation. Trends in Genetics, 6, 182–187.

Gopalakrishnan, S. (2008). Comparison of heavy metal toxicity in life stages (sper- miotoxicity, egg toxicity, embryotoxicity and larval toxicity) of *Hydroides elegans*. Chemosphere, 71, 515-528.

Guilherme, S., Gaivão, I., Santos, MA., Pacheco, M. (2010). European eel (*Anguilla anguilla*) genotoxic and pro-oxidant responses following short-term exposure to Roundup®: a glyphosate-based herbicide. Mutagenesis, 25 (5):523–530.

Guilherme, S., Santos, M. A., Barroso, C., Gaivão, I., and Pacheco, M. (2012). Differential genotoxicity of Roundup® formulation and its constituents in blood cells of fish (*Anguilla anguilla*): Considerations on chemical interactions and DNA damaging mechanisms. Ecotoxicology, *21* (5), 1381 – 1390.

Guilherme, S., Santos, M. A., Gaivão, I., and Pacheco, M. (2014a). Are DNA-damaging effects induced by herbicide formulations (Roundup® and Garlon®) in fish transient and reversible upon cessation of exposure? Aquatic Toxicology, *155*, 213–221.

Guilherme, S., Santos, M.A., Gaivão, I., Pacheco, M. (2014b). DNA and chromosomal damage induced in fish *(Anguilla anguilla L*.) by aminomethylphosphonic acid (AMPA)-the major environmental breakdown product of glyphosate. Environmental Science and Pollution Research. Int. 21 (14), 8730-9.

Guilherme, S., Santos, M.A., Gaivão, I., Pacheco, M. (2015). Genotoxicity evaluation of the herbicide Garlon® and its active ingredient (triclopyr) in fish (*Anguilla anguilla L*.) using the comet assay. Environmental Toxicology, 30, 1073–1081.

Habsburgo-Lorena, A.S. (1979). Present situation of exotic species of crayfish introduced into spanish continental waters. In: Freshwater crayfish IV. Papers

from the fourth international symposium on freshwater crayfish, Pierre-J. Laurent, ed., Institut National de la Recherche Agronomique, 175-184.

Halliwell, B., Gutteridge, JMC. (1999). Free radicals in biology and medicine, 3rd edn. Oxford University Press, New York.

Hanawalt, PC., Ford, JM., Lloyd, DR. (2003) Functional characterization of global genomic DNA repair and its implication for cancer. Mutation Research, 544, 107-114.

Hamilton, M. L., Van Remmen, H., Drake, J. a, Yang, H., Guo, Z. M., Kewitt, K., Richardson, A. (2001). Does oxidative damage to DNA increase with age? Proceedings of the National Academy of Sciences of the United States of America, 98 (18), 10469–74.

Hardersen, S., and Wratten, S. D. (2000). Sensitivity of aquatic life stages of *Xanthocne- mis zealandica* (Odonata: Zygoptera) to azinphos-methyl and carbaryl. New Zealand Journal of Marine and Freshwater Research. , 34 (1), 117-123.

Hartmann, A., Agurell, E., Beevers, C., Smith, A., Speit, G., Thybaud, V., & Tice, R. R. (2003). Recommendations for conducting the in vivo alkaline Comet assay,Mutagenesis *18* (1), 45–51.

Hayashi, M., Ueda, T., Uyeno, K., Wada, K., Kinae, N., Saotome, K., Tanaka, N., Takai, A., Sasaki, YF., Asano, N., Sofuni, T., Ojima, Y. (1998). Development of genotoxicity assay systems that use aquatic organisms. Mutation Research, 399 (2),125-133.

Helfrich, LA., Weigmann, DL., Hipkins, P., and Stinson., ER (1996). Pesticides and aquatic animals: A guide to reducing impacts on aquatic systems. Virginia Cooperative Extension.

Helleday, T., Lo, J., van Gent, D. C., and Engelward, B. P. (2007). DNA double-strand break repair: from mechanistic understanding to cancer treatment. DNA Repair, *6* (7), 923–35.

Hoang, T. C., and Klaine, S. J. (2007). Influence of organism age on metal toxicity to *Daphnia magna*. Environmental Toxicology and Chemistry. , 26 (6), 1198-1204.

Hobbs, H.H.Jr. (1984). On the distribution of the Crayfish genus Procambarus (Decapoda: Cambaridae). Journal of Crustacean Biology, 4 (1), 12-24.

Hobbs, H.H., and J.P. Jass (1989). The Crayfishes & Shrimp of Wisconsin (Cambaridae, Palaemonidae). Milwakee public museum eds., 140 pag.

Hobbs, H.H.III, J.P., jass and J.V., Huner. (1989). A review of global crayfish introductions with particular emphasis on two North American species (*Decapoda, Cambaridae*). Crustaceana, 56 (3), 299-316.

Holdich, D.M. (1987). The dangers of introducing alien animals with particular reference to crayfish. In: Freshwater crayfish VII. Papers from the seventh international symposium of Astacology, Pierre Goeldlin de Tifenau, ed., Musée Zoologique Cantonal.

Holdich, D.M., and R.S., Lowery (1988). Freshwater crayfish. Biology, management and exploitation. In: Freshwater crayfish. Biology management and exploitation, 1st edn, D.M. Holdich and R.S. Lowery, ed., Croom Helm. London and Sidney, 498 pp.

Holdich, D. M., J. D. Reynolds, C., Souty-Grosset and P. J., Sibley. (2009). A review of the ever increasing threat to European crayfish from non-indigenous crayfish species. Knowledge and Management of Aquatic Ecosystems, 11, 394–395.

Hoeijmakers JH. (2001) Genome maintenance mechanisms for preventing cancer. Nature, 411, 366-374.

Huner, J.V., and J.W. Avault Jr. (1979). Introductions of *Procambarus spp*. - A report to the introductions committee of the international Association of Astacology, fourth biennal meeting Thonon-les-Bains, France, 28-31 August 1978. In: Freshwater crayfish IV. Papers from the fourth international

symposium on freshwater crayfish, Pierre-J. Laurent, ed., Institut National de la Recherche Agronomique, 191-194.

Iarmarcovai, G., Bonassi, S., Sari-Minodier, I., Baciuchka-Palmaro, M., Botta, A., & Orsière, T. (2007). Exposure to genotoxic agents, host factors, and lifestyle influence the number of centromeric signals in micronuclei: A pooled re-analysis. Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis, *615* (1-2), 18–27.

Instituto Superior Técnico - IST/ INAG (2001). Limites dos estuaries de Portugal. (http://194.65.82.105/Estuarios/Inicio/frame_page.htm).

Jabusch, TW., Tjeerdema, RS. (2005) Partitioning of penoxsulam, a new sulfonamide herbicide. Journal of Agricultural and Food Chemistry, 53, 7179–7183.

Jabusch, T.W., Tjeerdema, R.S. (2006a). Photodegradation of penoxsulam. Journal of Agricultural and Food Chemistry, 54, 5958–5961.

Jabusch, T.W., Tjeerdema, R.S. (2006b). Microbial degradation of penoxsulam in flooded rice field soils. Journal of Agricultural and Food Chemistry, 54, 5962–5967.

Jackson, S. P. (2002). Sensing and repairing DNA double-strand breaks. Carcinogenesis, *23* (5), 687–696.

Jha, A.N. (2008). Ecotoxicological applications and significance of the comet assay. Mutagenesis, 23, 207–221.

Kennedy, A. J., Millward, R. N., and Steevens, J. A., Lynn, and. Perry, K.D.J.W. (2006). Relative sensitivity of zebra mussel (*Dreissena polymorpha*) life stages to two copper sources. Journal of Great Lakes Research., 32, 596-606.

Kirsch-Volders, M., Aardema, M., Elhajouji, A. (2000). Concepts of thresholds in mutagenesis and carcinogenesis. Mutation Research, 464, 3-11.

Kirsch-Volders, M., Vanhauwaert, A., Eichenlaub-Ritter, U., and Decordier, I. (2003). Indirect mechanisms of genotoxicity. Toxicology Letters, 140-141, 63–74.

Klerks, P. L. (1999). Acclimation to contaminants by the Grass Shrimp. Ecotoxicology, 277–286.

Klerks, P. L., and Moreau, C. J. (2001). Heritability of resistance to individual contaminants and to contaminant mixtures in the sheepshead minnow (*Cyprinodon Variegatus*). Environmental Toxicology and Chemistry, *20* (8), 1746–1751.

Khan, A.T., Forester, D.M., Mileke, H.W. (1995). Heavy metal concentrations in two populations of crayfish. Veterinary and Human Toxicology, 37, 426–428.

Klobučar, G. I. V., Malev, O., Šrut, M., Štambuk, A., Lorenzon, S., Cvetković, Ž., Maguire, I. (2012). Genotoxicity monitoring of freshwater environments using caged crayfish (*Astacus leptodactylus*). Chemosphere, *87* (1), 62–7.

Kolle, Susanne. (2012). "Genotoxicity and Carcinogenicity". BASF The Chemical Company. http://www.alternative-methods.basf.com.

Kopciuch, R., Berecka, B., Bartoszewicz, J., and Buszewski, B. (2004). Some considerations about bioindicators in environmental monitoring, *13* (5), 453–462.

Kouba, A., Miloš,Buřič, Pavel., Kozák., (2010). Bioaccumulation and effects of heavy metals in crayfish: a Review. Water Air & Soil Pollution, 211, 5–16.

Lee, R. F., and Steinert, S. (2003). Use of the single cell gel electrophoresis/ comet assay for detecting DNA damage in aquatic (marine and freshwater) animals. Mutation Research, 544, 43–64.

Lodish, H., Berk, A., Matsudaira, P., Kaiser, CA., Krieger, M., Scott, MP., Zipursky, SL., and Darnell, J.(2004). Molecular Biology of the Cell, p 963. WH Freeman: New York, NY. 5th ed.

Marques, C.R., Gonçalves, A.M.M., Pereira, R. and Gonçalves, F. (2012). Ecotoxicological effects of Mikado® and Viper® on algae and daphnids. Environmental Toxicology, 27, 685–699.

Marques, A., Custódio, M., Guilherme, S., Gaivão, I., Santos, M.A., Pacheco, M. (2014a). Assessment of chromosomal damage induced by a deltamethrin-based insecticide in fish (*Anguilla anguilla L.*) - a follow-up study upon exposure and post-exposure periods. Pesticide Biochemistry and Physiology, 113, 40–6.

Marques, A., Guilherme, S., Gaivão, I., Santos, M.A., Pacheco, M. (2014b). Progression of DNA damage induced by a glyphosate-based herbicide in fish (*Anguilla anguilla*) upon exposure and post-exposure periods--insights into the mechanisms of genotoxicity and DNA repair. Comparative Biochemistry and Physiology. Part C: Toxicology & Pharmacology, 166, 126–33.

Marques, A., Rego, A., Guilherme, S., Gaivão, I., Santos, M.A., Pacheco, M. (2016). Evidences of DNA and chromosomal damage induced by the mancozeb-based fungicide Mancozan® in fish (*Anguilla anguilla L.*). Pesticide Biochemistry and Physiology. 133, 52-58.

Marshal and Williams. (1972). The freshwater crayfish (*Astacus sp*). In: Textbook of zoology: Invertebrates, Marshal and Williams, ed., Macmillan Press: London, 493-511.

Masiá, A., Campo, J., Vázquez-Roig, P., Blasco, C., Picó, Y. (2013) Screening of currently used pesticides in water, sediments and biota of the Guadalquivir River Basin (Spain). Journal of Hazardous Materials, 263, 95–104.

Mitchelmore, C., Chipman, J. (1998). DNA strand breakage in aquatic organisms and the potential value of the comet assay in environmental monitoring. Mutation Research, 399, 135–147.

Mohammed, A. (2013). Why are early life stages of aquatic organisms more sensitive to toxicants than adults? New Insights into Toxicity and Drug Testing, 49–62.

Monserrat, J. M., Martínez, P. E., Geracitano, L. A., Amado, L. L., Martins, C. M. G., Pinho, G. L. L., Chaves, I. S., Ferreira-Cravo, M., Ventura-Lima, J., and Bianchini, A. (2007). Pollution biomarkers in estuarine animals: Critical review and new perspectives. Comparative Biochemistry and Physiology, 146, 221 – 234.

Mourgues, S., Lomax, ME., O'Neill, P. (2007) Base excision repair processing of a basic site/single-strand break lesions within clustered damage sites associated with XRCC1Deficiency. Nucleic Acids Res 35, 7676-7687.

Murussi, C. R., Thorstenberg, M. L., Leitemperger, J., Costa, M., Clasen, B., Santi, A.,Loro, V. L. (2014). Toxic effects of penoxsulam herbicide in two fish species reared in southern Brazil. Bulletin of Environmental Contamination and Toxicology, 92 (1), 81–4.

Nagarathna, P. K. M., Johnson Wesley, M., Sriram Reddy, P., and Reena, K. (2013). Review on genotoxicity, its molecular mechanisms and prevention. International Journal of Pharmaceutical Sciences Review and Research, 22 (1), 236–243.

Natarajan AT. (1993). Mechanisms for induction of mutations and chromosome alterations. Environmental Health Perspectives, 101, 225-229.

Östling, O., Johanson, KJ. (1984). Microelectrophoretic study of radiation induced DNA damages in individual mammalian cells. Biochemical and Biophysical Research Communications, 123, 291–298.

Ownby, D. R., Newman, M. C., Mulvey, M., Vogelbein, W. K., Unger, M. A., & Arzayus, L. F. (2002). Fish (*Fundulus heteroclitus*) populations with different exposure histories differ in tolerance of creosote-contaminated sediments. Environmental Toxicology and Chemistry, *21* (9), 1897–1902.

Pacheco, M., Santos, MA. (1997). Induction of EROD activity and genotoxic effects by polycyclic aromatic hydrocarbons and resin acids on juvenile eel *Anguilla anguilla L*. Ecotoxicology and Environmental Safety, 38, 252-25.

Pellegri, V., Gorbi, G., and Buschini, A. (2014). Comet assay on *Daphnia magna* in eco-genotoxicity testing. Aquatic Toxicology, *155*, 261–268.

Picado, A., Bebianno, M.J., Costa, M.H. (2007). Hydrobiologia, 587: 79.

Rahman, MF., Mahboob, M., Danadevi, K., Saleha Banu, B., Grover, P. (2002). Assessment of genotoxic effects of chloropyriphos and acephate by the comet assay in mice leucocytes. Mutation research/genetic toxicology and environmental mutagenesis, 516 (1-2), 139–147.

Rastogi, R. P., Kumar, A., Tyagi, M. B., and Sinha, R. P. (2010). Molecular mechanisms of ultraviolet radiation-induced DNA damage and repair, 2010.

Regoli, F. (2000). Total oxyradical scavenging capacity (TOSC) in polluted and translocated mussels: A predictive biomarker of oxidative stress. Aquatic Toxicology, 50 (4), 351–361.

Roberts, D.W., Knuteson, J.A., Jackson, R. (2003). The dissipation of penoxsulam in flooded rice fields. In: Pesticides in air, plant, soil and water systems; XII Symposium pesticide chemistry, Piacenza, Italy. Institute of Agricultural and Environmental Chemistry of the Catholic University, Piacenza, Italy, pp. 349–357.

Robertson KD. (2001). DNA methylation, methyltransferases, and cancer. Oncogene 20 (24): 3139–3155.

Rodrigues, BN., Almeida, FS. (2005). Guia de herbicidas, 5th edn. IAPAR, Londrina, p 648.

Sancar, A., Lindsey-Boltz, L. a., Unsal-Kaçmaz, K., and Linn, S. (2004). Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. Annual Review of Biochemistry, *73*, 39–85.

Sanders, B.M., Goering, P.L., Jenkins, K., (1996). The role of general and metal-specific cellular responses in protection and repair of metal-induced damage: stress proteins and metallothioneins. In Chang, L.W. (Ed.), Toxicology of Metals. CRC Press, Boca Raton, FL, pp. 165–187.

Santos, S., Vilar, V. J. P., Alves, P., Boaventura, R. a R., and Botelho, C. (2013).Water quality in Minho/Miño River (Portugal/Spain). Environmental Monitoring and Assessment, 185 (4), 3269–81.

Scalon, MCS., Rechenmacher, C., Siebel, AM., Kayser, ML., Rodrigues, MTAS., Maluf, SW., Silva, LB. (2010). Evaluation of Sinos river water genotoxicity using the comet assay in fish. Brazilian. Journal of Biology, 70, 1217–1222.

Schilderman, P.A.E.L., Moonen, E.J.C., Maas, L.M., Welle, I., Kleinjans, J.C.S. (1999). Use of crayfish in biomonitoring studies of environmental pollution of the river Meuse. Ecotoxicol. Ecotoxicology and Environmental Safety, 44, 241–252.

Schmieder, P. K., Jensen, K. M., Johnson, R. D., and Tietge, J. E. (2000). Comparative sensitivity of different life-stages of medaka and salmonid fishes to 2,3,7,8-TCDD. Presented at International symposium on endocrine-disrupting substances testing in Medaka, Nagoya, Japan, March , 17-20.

Sedgwick, B., Bates, PA., Paik, J., Jacobs, SC., Lindahl, T. (2007). Repair of alkylated DNA: recent advances. DNA Repair, 6, 429-442.

Sevatdal, S., and Horsberg, T. E. (2003). Determination of reduced sensitivity in sea lice (*Lepeophtheirus salmonis* Krøyer) against the pyrethroid deltamethrin using bioassays and probit modelling. Aquaculture, *218* (1-4), 21–31.

Shao, B., Zhu, L., and Dong, M. (2012). DNA damage and oxidative stress induced by endosulfan exposure in zebrafish (*Danio rerio*). Ecotoxicoly, 1533–1540.

Singh, NP., McCoy, MT., Tice, RR., Schneider, EL. (1988). A simple technique for quantitation of low levels of DNA damage in individual cells. Experimental Cell Research,175, 184–191.

Speit, G., Schütz, P. Bonzheim, I., Trenz, K., Hoffmann, H., (2004). Sensitivity of the FPG protein towards alkylation damage in the comet assay. Toxicology Letters, 146, 151–158.

Speit, G., Schütz, P. (2008). Mutation Research / Genetic Toxicology and Environmental Mutagenesis The effect of inhibited replication on DNA migration in the comet assay in relation to cytotoxicity and clastogenicity. Mutation Research 655, 22–27.

Straalen V. (2003*). "Ecotoxicology becomes Stress Ecology".* Environmental science and technology. 37,324–329.

Stoiber, T., Bonacker, D., Bohm, K., Bolt, H., Their, R., Degen, G., Unger, E. (2004). Disturbed microtubule function and induction of micronuclei by chelate complexes of mercury (II). Mutation Research, 563,97-106.

Suárez-Serrano, A., Alcaraz, C., Ibanez, C., Trobajo, R., and Barata, C. (2010a). *Procambarus clarkii* as a bioindicator of heavy metal pollution sources in the lower Ebro River and Delta. Ecotoxicology and Environmental Safety, 73, 280–286.

Suárez-Serrano, A., Ibáñez, C., Lacorte, S., Barata, C. (2010b). Ecotoxicological effects of rice field waters on selected planktonic species: comparison between conventional and organic farming. Ecotoxicology, 19, . 1523–1535.

Taylor, P., and Weis, J. S. (2010). Tolerance to environmental contaminants in the Mummichog, *Fundulus heteroclitus*. Human and ecological risk assessment : An international journal, 37–41.

Taketomi, Y., M. Murata and M. Miyawaki (1990). Androgenic gland and secondary sexual characters in the Crayfish *Procambarus clarkii*. Journal of Crustacean Biology, 10 (3), 492-497.

Van Gent, DC., Hoeijmakers, JHJ., Kanaar, R., (2001). Review: Chromosomal stability and the DNA double-stranded break connection. Nature Reviews Genetics 2, 196-206.

Van Steeg, H. (2009). The role of nucleotide excision repair and loss of p53 in mutagenesis and carcinogenesis. Toxicology Letters 120, 209-219.

Vioque-Fernández, A., Alves de Almeidab, E., Ballesteros., J., García-Barrera, T., GómezAriza, J.L., López-Barea, J. (2007a). Doñana National Park survey using crayfish (*Procambarus clarkii*) as bioindicator: esterase inhibition and pollutant levels. Toxicology Letters 3, 260–268.

Vioque-Fernándeza, A., Alves de Almeidab, E., López-Barea, J. (2007b). Esterases as pesticide biomarkers in crayfish (*Procambarus clarkii*, Crustacea): tissue distribution, sensitivity to model compounds and recovery from inactivation. Comparative Biochemistry and Physiology. Part C: Toxicology & Pharmacology 3, 404–412.

Vioque-Fernándeza, A., Alves de Almeidab, E., López-Barea, J. (2009a). Assessment of Doñana National Park contamination in *Procambarus clarkii*: integration of conventional biomarkers and proteomic approaches. Science of the Total Environment, 407, 1784–1797.

Vioque-Fernándeza, A., Alves de Almeidab, E., López-Barea, J. (2009b). Biochemical and proteomic effects in *Procambarus clarkii* after chlorpyrifos or carbaryl exposure under sublethal conditions. Biomarkers, 5, 299–310.

Washington State Department of Ecology (2012). Environmental impact statement for penoxsulam, imazamox, bispyribac-sodium, flumioxazin, and carfentrazone- ethyl: Addendum to the Final supplemental environmental impact statement for freshwater aquatic plant Management final.

Weston, D. P., Poynton, H. C., Wellborn, G. A., Lydy, M. J., Blalock, B. J., Sepulveda, M. S., and Colbourne, J. K. (2013). Multiple origins of pyrethroid insecticide resistance across the species complex of a nontarget aquatic

crustacean, Hyalella azteca. Proceedings of the National Academy of Sciences, 110 (41), 16532–16537.

Weber, L., Carvalho, L., Silva, V., Beraldini, N., and Souza, V. (2013). Genotoxic effects of the water-soluble fraction of heavy oil in the brackish / freshwater amphipod *Quadrivisio aff . lutzi* (Gammaridea) as assessed using the comet assay. Ecotoxicology, 642–655.

Wilcosky. T., Raynard., S. (1990). Biological markers in epidemiology. Oxford University Press, USA.

Williams, L. M., and Oleksiak, M. F. (2008). Signatures of selection in natural populations adapted to chronic pollution. BMC Evolutionary Biology, *8* (1), 282.

Wirgin, I., and Waldman, J. R. (2004). Resistance to contaminants in North American fish populations. Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis, 552 (1-2), 73–100.

Yüzbaşioğlu, D., Ünal, F., and Sancak, C. (2009). Genotoxic effects of herbicide Illoxan (Diclofop-Methyl) on *Allium cepa L* .Turkish Journal of Biology *33*, 283–290.

Zar, J. (1996). Biostatistical analysis., Prentice Hall International Inc., New Jersey.

Zhang, W., Huang, L., and Wang, W. X. (2012). Biotransformation and detoxification of inorganic arsenic in a marine juvenile fish *Terapon jarbua* after waterborne and die- tborne exposure. Journal of Hazardous Materials, 221-222,162.

Zheng, W., Lifen, J., Jiliang, He., Jianlin, L., Baohong, W., Hongping, D.(2005). DNA repair capacity of peripheral lymphocytes from cancer patients with UVC challenge and bleomycin challenge test. Mutagenesis , 20, 271-277.